Signalling and expression of the Ang-Tie pathway in tumor vasculature

Anita Lampinen

Research Programs Unit
Translational Cancer Biology Program
&
Doctoral Programme in Biomedicine

Faculty of Medicine
University of Helsinki
Finland

Academic dissertation

To be presented for public examination with the permission of the Faculty of Medicine, University of Helsinki, in Lecture Hall 3, Biomedicum Helsinki 1 (Haartmaninkatu 8, Helsinki), on December 15th 2016, at 12 noon.

Thesis supervisor

Docent, Ph.D. Pipsa Saharinen
Wihuri Research Institute
Translational Cancer Biology Program
Research Programs Unit
Faculty of Medicine
University of Helsinki
Finland

Reviewers appointed by the Faculty

Prof. M.D., Ph.D. Peppi Karppinen
Faculty of Biochemistry and Molecular Medicine
University of Oulu
Finland

and

Prof. M.D., Ph.D. Tom Böhling
Medicum
Haartman Institute
Department of Pathology
University of Helsinki
Finland

Opponent appointed by the Faculty

Associate Prof., Docent, Ph.D. Anna Dimberg
Department of Immunology, Genetics and Pathology
Uppsala University
Sweden

ISBN 978-951-51-2758-7 (paperback)
ISBN 978-951-51-2759-4 (PDF)
ISSN 2342-3161
http://ethesis.helsinki.fi

Unigrafia Oy
Helsinki 2016

Sisu alkaa siellä, missä sinnikkyys ja periksiantamattomuus loppuvat. Sisu on henkisen kestävyyden ja rikkoutumattomuuden toinen aalto, sekä toiminnan ääritilanteessa mahdollistava psyykkinen voimavara.

-Emilia Lahti

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LIST OF PUBLICATIONS

This thesis is based on the following publications and will be referred in the text by their Roman numerals (I-IV). The original publications have been reprinted with the permission of the publisher.

I. Rautiola J*, **Lampinen A*,** Mirtti T, Ristimäki A, Joensuu H, Bono P and Saharinen P. Association of Angiopoietin-2 and Ki-67 Expression with Vascular Density and Sunitinib Response in Metastatic Renal Cell Carcinoma**.** Plos One 11(4):e0153745, 2016. *Equal contribution.

II. **Lampinen A**, Virman J, Bono P, Luukkaala T, Sunela K, Kujala P, Saharinen P and Kellokumpu-Lehtinen P-L. Novel angiogenesis markers as long-term prognostic factors in renal cell cancer patients. Clinical Genitourinary Cancer 2016 Jul [Epub ahead of print].

III. Emilia A. Korhonen*, **Anita Lampinen***, Hemant Giri, Andrey Anisimov, Minah Kim, Breanna Allen, Shentong Fang, Gabriela D'Amico, Tuomas Sipilä, Marja Lohela, Tomas Strandin, Antti Vaheri, Seppo Ylä-Herttuala, Gou Young Koh, Donald M. McDonald, Kari Alitalo# and Pipsa Saharinen#. Tie1 controls angiopoietin function in vascular remodeling and inflammation. Journal of Clinical Investigation**.** 126(9):3495-510, 2016. * # Equal contribution.

IV. Holopainen T, Saharinen P, D'Amico G, **Lampinen A**, Eklund L, Sormunen R, Anisimov, A, Zarkada G, Lohela M, Heloterä H, Tammela T, Benjamin LE, Ylä-Herttuala S, Leow CC, Koh GY and Alitalo K. Effects of angiopoietin-2-blocking antibody on endothelial cell-cell junctions and lung metastasis. Journal of the National Cancer Institute. 104(6):461-475, 2012.

Publication I appears in the doctoral thesis of Dr. Juhana Rautiola (2015, University of Helsinki).

Publication II appears in the doctoral thesis of Dr. Juha Virman (2016, University of Tampere).

Publication IV appears in the doctoral thesis of Dr. Tanja Holopainen (2016, University of Helsinki).

ABBREVIATIONS

Ab	antibody
Akt	a serine/threonine kinase
Ang1	angiopoietin-1, also termed Angpt1
Ang2	angiopoietin-2, also termed Angpt2
BEC	blood vascular endothelial cell
BM	basement membrane
CAIX	carbonic anhydrase-9
CBR	clinical benefit rate
CCD	coiled-coil domain
ccRCC	clear cell renal cell carcinoma
Comp	cartilage oligomeric matrix protein
EC	endothelial cell
ECM	extracellular matrix
EGF	epidermal growth factor
ESAM	endothelial cell-selective adhesion molecule
ESM-1	endothelial cell specific molecule 1
FAK	focal adhesion kinase
FLIM	fluorescence lifetime imaging microscopy
Flk-1	Fetal liver kinase 1
FN III	fibronectin type III repeats
Foxo1	the Forkhead box protein O1
FRET	fluorescence resonance energy transfer
HGF	hepatocyte growth factor
HIF	hypoxia-inducible factor
ICAM	intercellular adhesion protein
IF	immunofluorescence
Ig	immunoglobulin
IHC	immunohistochemistry
IL	interleukin
IP	immunoprecipitation
JAM	junction adhesion molecule
KDR	kinase insert domain-containing receptor
LPS	lipopolysaccharide
miRNA	micro RNA
MAPK	mitogen activated protein kinase
MMP	matrix metalloproteinase
mRCC	metastatic renal cell carcinoma
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MVD	microvessel density
NRP-1	neuropilin-1
OS	overall survival
ORR	objective response rate
PECAM-1	the platelet endothelial cell adhesion molecule-1
PKC	protein kinase C
PD	progressive disease

PDGF	plateled-derived growth factor
PFS	progression-free survival
PHD	prolyl hydroxylase
PlGF	placental growth factor
PMA	phorbol myristate acetate
PR	partial response
pRCC	papillary renal cell carcinoma
Rap1	Ras-proximate-1 or Ras-related-protein 1
RCC	renal cell carcinoma
ROCK	Rho-associated protein kinase
ROI	region of interest
RTK	receptor tyrosine kinase
SCD	super-clustering domain
SD	stable disease
SGK1	serine/threonine-protein kinase
shRNA	short hairpin ribonucleic acid
SMC	smooth muscle cell
SNP	single nucleotide polymorphism
sTie	soluble Tie
TEM	Tie2 expressing monocyte/macrophage
TIMP	tissue inhibitor of metalloproteinase or TIMP metallopeptidase inhibitor
TKI	tyrosine kinase inhibitor
TNF-α	tumor necrosis factor-α
TMA	tissue microarray
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VE-PTP	Receptor-type tyrosine-protein phosphatase beta
VHL	von Hippel-Lindau
wAMD	wet age-related macular degeneration
WB	Western blot
WT	wild type
ZO-1	zonula occludens-1

TIIVISTELMÄ

Verisuonten uudismuodostus eli angiogeneesi on tärkeä prosessi yksilönkehityksen aikana. Myös monissa eri sairauksissa, kuten syövässä, angiogeneesi tuottaa verisuonia, jotka ylläpitävät syöpäkasvaimen kasvua ja mahdollistavat etäpesäkkeiden muodostumista muualle kudoksiin (metastaasi). Vaskulaariendoteelikasvutekijä (VEGF) ja angiopoietiinikasvutekijät (Ang1 ja Ang2) säätelevät veri- ja imusuonten muodostusta suonen sisäpinnan endoteelisolujen reseptorityrosiinikinaasien (VEGF-reseptori ja angiopoietiinireseptorit Tie1 ja Tie2) välityksellä. VEGF- ja Ang2-kasvutekijät lisäävät verisuonten versomista ja läpäisevyyttä, kun taas Ang1 aktivoi Tie2-reseptorin endoteelisolujen välisissä liitoksissa, mikä vähentää endoteelin läpäisevyyttä. Verisuonten kasvua estäviä lääkkeitä, jotka estävät VEGF-kasvutekijän toimintaa (anti-angiogeneesilääkkeet), on käytetty useiden syöpien hoidossa noin 10 vuoden ajan. Viime vuosien aikana levinneen eli metastasoivan munuaissyövän hoidoissa on saatu tuloksia lääkkeillä, jotka estävät mm. VEGF-kasvutekijäreseptoria ja VEGF-kasvutekijän aiheuttamaa angiogeneesiä. Usein hoidon aloituksen jälkeen syöpä kuitenkin uusiutuu. Tämän takia uusia Ang-Tie kasvutekijäreittiin kohdennettuja lääkkeitä on kehitteillä. Lisäksi on pyritty löytämään ns. biomarkkereita, joiden perusteella hoidot voitaisiin kohdentaa niistä parhaiten hyötyville syöpäpotilaille.

Tässä tutkimuksessa tutkittiin molekyylitason mekanismeja, jotka säätelevät Ang-Tie-signaalinvälitysreitin toimintaa verisuonien endoteelisolujen välisissä liitoksissa ja vuotavassa syöpäkasvaimen verisuonistossa, sekä Ang2-kasvutekijän ilmentymistä metastasoivan munuaissyöpäkasvaimen primärituumorissa ja sen potentiaalia biomarkkerina anti-angiogeneesihoidoissa.

Tässä tutkimuksessa osoitettiin, uusi funktio Tie1-reseptorille. Havaitsimme, että angiopoietiinit aktivoivat Tie2-reseptoria Tie1-reseptorin välityksellä, ja tämä vuorovaikutus ja reseptorien aktivaatio on riippuvainen $\alpha_5\beta_1$-integriinistä. Tie1-reseptorin poisto endoteelisoluista shRNA-välitteisesti vähensi Tie2-reseptorin aktivaatiota ja aiheutti Tie2-reseptorin siirtymisen pois solun pinnalta. FRET, FLIM ja TCSPC-FLIM mikroskopiaa käyttämällä osoitettiin Tie1- ja Tie2- reseptorien interaktio endoteelisolujen välisissä liitoksissa, ja tämä interaktio oli riippuvainen β_1-integriinistä. Estämällä β_1-integriinin ilmentyminen saatiin estettyä myös Tie reseptorien angiopoietiinivälitteinen aktivaatio, ja aktivoituneen Tie reseptorin käynnistämät signalointireitit. Lisäksi Tie1-poistogeenisessa hiirimallissa osoitettiin, että Tie1 reseptori vaaditaan Ang1- ja Ang2- väitteisiin signalointiin ja verisuonien uudismuodostumiseen.

Ang2-kasvutekijän määrä kohoaa seerumissa useissa eri sairauksissa, kuten syövässä. Tässä tutkimuksessa tutkittiin Ang2 vaikutusta etäpesäkkeiden muodostumiseen hiiren tuumorimalleissa. Tutkimuksessa osoitettiin, että Ang2-kasvutekijän siirtogeenivälitteinen ilmentäminen hiiressä lisäsi etäpesäkkeiden muodostumista keuhkoihin. Vastaavasti, Ang2-kasvutekijän estäminen vasta-aineella esti etäpesäkkeiden muodostumista ja paransi endoteelisolujen välisiä liitoksia. Soluviljelyssä endoteelisoluissa osoitettiin, että Ang2-kasvutekijän toimintaa estävät

vasta-aineet johtivat Ang2-Tie2 signalointikompleksin siirtymisen solun pinnalta solun sisälle, estäen tämän toimintaa.

Korkeat seerumin Ang2 tasot ennustavat lyhentynyttä elinikää useissa syövissä, mutta Ang2-kasvutekijän ilmentymistä syöpäkasvaimessa ja sen mahdollista korrelaatiota kliiniseen hoitovasteeseen anti-angiogeneesihoitojen aikana ei ole tutkittu. Tässä tutkimuksessa määritettiin immunohistokemiallisella värjäyksellä Ang2-kasvutekijän ilmentyminen levinneen munuaissyövän primärituumoreista, sekä verisuonten että jakautuneiden syöpäsolujen määrät. Ang2 ilmeni kasvaimen verisuonien endoteelisoluissa, ja korkea verisuonten ja Ang2-kasvutekijän määrä lisäsivät potilaiden todennäköisyyttä hyötyä sunitinib-hoidosta (P = 0.002). Matala jakaantuvien syöpäsolujen määrä ennusti merkittävästi potilaiden kokonaiselossaoloajan pidentymistä (P = 0.015). Toisessa potilasaineistossa, jossa potilaat eivät saaneet anti-angiogeneesihoitoa, korkea Ang2 määrä kasvaimen verisuonistossa ennusti pidentynyttä potilaiden kokonaiselossaoloaikaa.

Yhteenvetona tämän tutkimuksen tulokset osoittavat, että Tie1- ja Tie2-reseptorien vuorovaikutus vaaditaan endoteelisolujen angiopoietiinivasteeseen ja verisuonien muokkaukseen, ja että Tie1-reseptori säätelee Tie2 liikkumista solussa. Osoitimme myös, että Ang2-kasvutekijää estävät vasta-aineet estävät Ang2 sitoutumista Tie2-reseptoriin, ja aiheuttavat Tie2-Ang2 -signaalikompleksin siirtymisen solun sisälle, estäen kompleksin toiminnan. Kokeellisissa syöpäkasvaimissa Ang2-vasta-aineen käyttö vähensi merkittävästi etäpesäkkeiden muodostumista keuhkoihin, mikä johtui osaltaan vasta-aineen verisuonia tiivistävästä vaikutuksesta. Lisäksi osoitimme, että Ang2 ilmentyy munuaissyöpäkasvaimen verisuonistossa ja korkea Ang2-pitoisuus ennustaa hoitovastetta sunitinib-hoidolle. Nämä tulokset tuovat uutta tietoa Ang-Tie systeemin toiminnasta endoteelisoluissa ja sen vaikutuksesta syöpäkasvaimen leviämiseen verisuoniston kautta.

ABSTRACT

Angiogenesis, the growth of new blood vessels, is essential for embryonic development, but it is also involved in numerous human diseases, including cancer. Vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs), as well as angiopoietin growth factors (Ang1, Ang2) together with their Tie1 and Tie2 tyrosine kinase receptors are essential regulators of angiogenesis and lymphangiogenesis. These pathways also control vessel permeability, for example in endotoxemia. VEGF-targeted therapies have been in clinical use for more than a decade, whereas Ang-Tie targeted drugs are in clinical development for cancer, as well as neovascular and edema causing eye diseases. Renal cell carcinoma (RCC) is a highly vascular tumor, which is notoriously resistant to chemotherapy and in the majority of the cases to immunotherapy. In recent years, blocking of VEGF-driven angiogenesis has become the choice of therapy in the metastatic form of RCC (mRCC). However, problems with anti-angiogenic therapies are associated with drug related resistance, and biomarkers that would predict patient responses are lacking.

In this study, we focused to uncover the molecular mechanisms of the Ang-Tie signalling system with relevance to tumor metastasis and endotoxemia. Second, we sought to investigate Ang2 expression in human mRCC, and its potential as a biomarker for anti-angiogenic therapies.

We found that Tie1 is essentially required for angiopoietin signalling in the mature vasculature and for agonistic activity of Ang2, which was lost in inflammation. Using fluorescence resonance energy transfer (FRET), fluorescence lifetime imaging microscopy (FLIM) and time correlated single photon counting (TCSPC)-FLIM, we demonstrated angiopoietin induced interactions between Tie1 and Tie2 receptors in a distance less than 10 nm in intact endothelial cells (ECs). Tie receptor interactions occurred preferentially in endothelial cell-cell (EC-EC) junctions, and were dependent on the EC -extracellular matrix (ECM) adhesion receptor, β_1-integrin. Short hairpin ribonucleic acid (shRNA) silencing of β_1-integrin inhibited also Tie receptor activation and downstream signalling. Using live cell imaging we found that Tie1 regulated Tie2 trafficking in ECs after angiopoietin stimulation. These results provide mechanistic explanations for the decreased angiopoietin signalling and vessel remodelling observed upon conditional deletion of Tie1 in the mouse endothelium. We also found, that Tie1 is essential for the agonist activity of Ang1 and autocrine Ang2 *in vivo* during vascular remodelling. In inflammatory conditions, the Tie1 ectodomain was rapidly cleaved resulting in decreased Ang1 agonist activity and complete loss of autocrine Ang2 agonist activity, leading to impaired Tie2 signalling.

Ang2 is induced by hypoxia in tumor ECs promoting angiogenesis. To understand Ang2 function in tumor progression, we investigated the effect of Ang2 on metastatic dissemination using tumor xenografts in mice. We found that Ang2 overexpression in transgenic mice decreased endothelial integrity in pulmonary capillaries and enhanced tumor metastasis to lungs, whereas lung metastasis was inhibited using Ang2-blocking antibodies (Abs). At the molecular level, Ang2-blocking Abs inhibited Ang2-Tie2

signalling by inducing internalization of the plasma membrane localized Ang2-Tie2 complexes.

Elevated circulating Ang2 levels have been reported to predict poor prognosis in certain human cancers, but the expression of Ang2 in the primary tumor or its significance for anti-angiogenic therapies have not been widely investigated. We developed a method to detect Ang2 in the vascular endothelium of human tumors. In the first patient cohort, we found that high pre-therapeutic Ang2 expression, especially when combined with high expression of endothelial marker CD31, was associated with a high clinical benefit rate (CBR) to first-line sunitinib therapy ($P = 0.002$). Low tumor cell proliferation marker Ki-67 expression was associated with significantly prolonged progression-free survival (PFS) ($P = 0.009$) and overall survival (OS) ($P = 0.015$). In the second cohort high Ang2 expression in tumor vasculature was associated with prolonged OS in patients who received no systemic anti-angiogenic therapies.

In summary, our results demonstrate that Tie1 is essential for angiopoietin signalling and vascular remodelling, by interacting with Tie2 and regulating the subcellular trafficking of Tie2. Second, a therapeutic anti-Ang2 Ab that promotes vessel integrity and inhibits tumor metastasis, works by inhibiting Ang2 binding to Tie2, and inducing the internalization of Ang2-Tie2 signalling complexes from ECs. Third, Ang2 is expressed in the vasculature of RCC tumors, correlating with clinical response to sunitinib and OS, calling for more thorough studies of Ang2 in human RCC. These results advance the current understanding of the function of the Ang-Tie pathway in the vasculature.

INTRODUCTION

Angiogenesis, the formation of new blood vessels, is essential for embryonic development and normal homeostasis, but it is also involved in numerous human diseases, such as in cancer. Tumor angiogenesis supports tumor growth and facilitates metastatic dissemination of tumor cells. Angiogenesis is a complex process, which is regulated by multiple growth factors and cytokines in concert with cell adhesion receptors, integrins. VEGFs and their receptors are master regulators of early blood and lymphatic vascular development. In addition, Ang1 and Ang2 growth factors regulate vascular development after mid-gestation via their endothelial receptor tyrosine kinases (RTKs) Tie2 and Tie1 (Augustin et al., 2009, Lohela et al., 2009, Eklund and Saharinen, 2013).

Ang1 acts as an agonist inducing translocation and activation of Tie2 in EC-EC contacts resulting in EC stabilization and survival and in EC-EC contacts regulating EC migration (Saharinen et al., 2008). Depending on the context, Ang2 may act as a weak agonist or antagonist of Tie2, inhibiting Ang1-mediated Tie2 activation by competing for Tie2 binding (Maisonpierre et al., 1997, Yuan et al., 2009). However, the molecular mechanisms that determine how Ang1 and Ang2 activate Tie2 resulting in different signalling outcomes are not well understood. Tie1 is an orphan receptor with no known ligand, but angiopoietins can activate Tie1 via Tie2 interaction (Saharinen et al., 2005). The molecular function of Tie1 in angiopoietin signalling and downstream signalling proteins activated by Tie1 are also not well established.

Ang2 expression is low under normal homeostasis, but elevated in numerous diseases, including cancer and inflammation (Eklund and Saharinen, 2013). High circulating Ang2 levels and increased Ang2 messenger ribonucleic acid (mRNA) expression have been found to predict for poor prognosis in breast cancer, melanoma and RCC (Sfiligoi et al., 2003, Helfrich et al., 2009, Wang et al., 2014). Also, Ang2 protein expression in the tumor tissue has also been reported (Sfiligoi et al., 2003), but the potential significance of Ang2 expression in response to targeted therapies has been only scarcely studied.

Ample preclinical evidence has shown that blocking the Ang-Tie system inhibits tumor growth and tumor angiogenesis. Thus, the Ang-Tie system has become a target for second-generation anti-angiogenic therapies, which are currently in clinical phase II-III trials in many human cancers (Monk et al., 2014, Atkins et al., 2015). However, the signalling mechanisms of the Ang-Tie system in the tumor vasculature are incompletely understood, and the expression of the Ang-Tie pathway components in human cancer has not been largely investigated.

RCC is a highly vascular tumor, which accounts for approximately 2-3 % of all adult malignancies, and has an increasing rate of incidence in many countries. RCC is highly resistant to chemotherapy and in the majority of the cases to traditional immunotherapy based on interferon-α. Increased knowledge of the molecular pathogenesis of RCC has established new targets in the disease management (Rini et

al., 2009). Sunitinib is a multi-targeted tyrosine kinase inhibitor (TKI) and widely used as a first-line therapy for mRCC. Previous studies have shown objective response rates (ORRs) to sunitinib in mRCC, however complete durable responses are rare (Motzer et al., 2006). To improve efficacy, investigational Ang1/Ang2 blocking peptibody has been combined in a clinical phase II RCC trial with sunitinib, and this combinational treatment suggested a potential clinical benefit (Atkins et al., 2015).

Current anti-angiogenic drugs, which inhibit the growth of new blood vessels, have revolutionized the treatment of many human cancers, however, complete responses are rare, and most patients are either refractory or relapse after a few months of treatment. Furthermore, no clear guidelines exist for patient- or tumor-specific selection of anti-angiogenic therapeutic agents, and so far no established biomarkers for antiangiogenic therapies are in clinical use. Therefore, novel treatment modalities for cancer are urgently needed, as well as knowledge of the biomarkers for the selection of patients, who will benefit from the anti-angiogenic therapy.

In this work, I have investigated signalling mechanisms of the Ang-Tie system, from the molecular level to the translational level, in order to better understand the mechanisms leading to vessel remodelling during inflammation, and in tumors and tumor metastasis. We show, that the orphan Tie1 is an essential component of the Ang-Tie2 signalling pathway, of Tie2 trafficking and that it also mediates Tie1-Tie2 interaction in EC-EC contacts in a β_1-integrin depended manner. Also, we show that a therapeutic anti-Ang2 Ab inhibits tumor growth and metastasis, and provide a mechanism of Ab action, supporting the concept that anti-Ang2 Abs may have potential in clinical cancer therapies. At the translational level, we show that high endothelial Ang2 expression in the primary RCC tumor alone or when combined with high microvessel density (MVD) was associated with clinical benefit to sunitinb in mRCC patients. Results from another cohort of RCC patients, who received no targeted therapies, demonstrate that very high endothelial Ang2 expression in RCC tumor was associated with better OS. These studies highlight the need for more thorough investigations of Ang2 expression in human cancer. These findings have the potential to facilitate the future therapeutic targeting of the Ang-Tie system and its use as a biomarker in human disease.

LITERATURE REVIEW

1. Blood vasculature

1.1. Structure and functions of the blood vascular system

Blood and lymphatic vasculatures form essential systems for the transport of substances in the body. The blood vasculature transports oxygen, nutrients, macromolecules and cells into distant tissues and back to pulmonary circulation. The arterial blood circulation, formed by arteries and arterioles is connected via capillaries to the venous system formed by venules and veins. ECs are essential building blocks of blood and lymphatic vessels, forming the innermost layer of the vessels that faces the vessel lumen. ECs are surrounded by a vascular basement membrane (BM), which they share with mural cells, including vascular smooth muscle cells (SMCs) embedded in connective tissue around arteries and veins and pericytes, which are found in postcapillary venules and venules (Baluk et al., 2005). The perivascular cells provide vessel stabilizing signal (reviewed in Jeltsch et al., 2013). Pericytes also participate in the maturation and remodelling of the blood vascular system and critically regulate the blood brain barrier (Benjamin et al., 1998, Gerhardt and Betsholtz, 2003, Armulik et al., 2010). Capillaries consist almost exclusively of ECs (reviewed in Jeltsch et al., 2013).

The formation of new blood vessels from pre-existing vessels, called angiogenesis, is an important process in both physiological and pathological conditions. During angiogenesis, quiescent ECs undergo a switch to an activated state, including upregulation of metalloproteases that degrade the ECM to enable EC migration to the surrounding space and EC proliferation (Kumar et al., 2014).

1.2. Growth factor regulation of vascular endothelial cells

1.2.1. VEGFs and VEGFRs

VEGFs (VEGF-A, -B, -C, and -D and placental growth factor, (PlGF) and their receptors (VEGFR1, -2, and -3) are essential regulators of angiogenesis (**Figure 1.**). The VEGF family members are secreted, homodimeric glycoproteins, which include several isoforms produced by alternative exon splicing (Houck et al., 1991, Tischer et al., 1991) and post-translational modifications (Xiong et al., 1998). VEGF-A (VEGF) is a ligand for VEGFR2 and VEGFR1, VEGF-B and PlGF for VEGFR1, VEGF-C and VEGF-D for VEGFR3, although the fully processed form of VEGF-C can also bind to VEGFR2.

VEGF exists in six isoforms ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{162}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, $VEGF_{206}$ (Houck et al., 1991, Park et al., 1993, Poltorak et al., 1997, Vempati et al., 2014), which are generated via alternative splicing. VEGF expression is induced by the hypoxia-inducible factor-1α (HIF-1α) in hypoxic conditions (Brogi et al., 1996). VEGF is essential for normal blood vascular growth during embryonic

development, and lack of already one VEGF allele is lethal (Ferrara et al., 1996). VEGF is the major regulator of vasculogenesis and angiogenesis and it binds to VEGFR2 and VEGFR1 (de Vries et al., 1992, Terman et al., 1992, Millauer et al., 1993, Quinn et al., 1993). Ligand binding to VEGFR2 stimulates its tyrosine phosphorylation leading to a strong angiogenic response (Waltenberger et al., 1994). Instead, VEGF binding to VEGFR1 induces a weak tyrosine kinase activity, and is required to supress excess angiogenesis (Ferrara, 2004).

VEGF-B is expressed in tissues with high metabolic activity, such as in skeletal muscle and vascular SMCs, myocardium, brown adipose tissue, kidney and the brain (Olofsson et al., 1996, Lagercrantz et al., 1998, Aase et al., 1999, Bry et al., 2014). VEGF-B is structurally very similar to VEGF (Grimmond et al., 1996, Olofsson et al., 1996). VEGF-B has a splicing site in exon six and it is expressed as two isoforms (Grimmond et al., 1996, Olofsson et al., 1996), which both bind to VEGFR1 and Neuropilin-1 (NRP-1), but not to VEGFR2 and VEGFR3 (Olofsson et al., 1998, Makinen et al., 1999). On the contrary to VEGF, hypoxia does no induce VEGF-B expression (Enholm et al., 1997). VEGF-B is not a strong inducer of angiogenesis (reviewed in Bry et al., 2014). It stimulates weakly sprouting angiogenesis, and overexpression of VEGF-B in the skin increased minimally blood vessel density (Karpanen et al., 2008). *Vegf-b* deficient mice and rats do not have pronounced vascular or developmental defects (Aase et al., 2001, Kivela et al., 2014). Instead, transgenic expression of *Vegf-b* induced the growth of coronary arteries and enlargement of myocardial capillaries (Karpanen et al., 2008, Bry et al., 2010). Both mice and rats expressing transgenic *Vegf-b* present cardiac hypertrophy and capillary enlargement (Karpanen et al., 2008, Li et al., 2008, Bry et al., 2010). In addition, overexpression of *Vegf-b* in mice increased the growth of new blood vessels in the infarcted and ischemic border zone in the heart (Li et al., 2008). VEGF-B is upregulated in certain cancers, such as RCC and colorectal cancer, but its function in human cancer remains unknown (Gunningham et al., 2001, Hanrahan et al., 2003).

VEGF-C is required for the development of lymphatic vessels, and it is also a major regulator of lymphangiogenesis in adults. In addition, fully proteolytically processed VEGF-C can induce neovascularization of mouse cornea (Cao et al., 1998). VEGF-C binds to and activates VEGFR3 (Joukov et al., 1996). VEGF-C is proteolytically cleaved resulting in high affinity binding of VEGF-C to VEGFR3 and to VEGFR2 (Joukov et al., 1997). Constitutive *Vegf-c* deficient mouse embryos die during embryonic period because of defective lymphatic vessel development leading to fluid accumulation in the tissue (edema). Mouse embryos heterozygous for *Vegf-c* have defective lymphatic vasculature and lymphedema, nevertheless they survive during embryonic development until adulthood (Karkkainen et al., 2004). VEGF-C is expressed by many tumor cells and tumor associated myeloid cells (reviewed in Karpanen and Alitalo, 2008). In tumors, VEGF-C expression induces lymphangiogenesis facilitating tumor cell intravasation into the vessel and lymphatic metastasis (Skobe et al., 2001).

VEGF-D is a second growth factor that stimulates lymphangiogenesis via binding to VEGFR3 and the NRP co-receptors, and it undergoes similar proteolytic cleavage as VEGF-C (Achen et al., 1998). VEGF-D can also promote tumor growth and angiogenesis by stimulating tumor lymphangiogenesis in mouse tumor models (Stacker et al., 2001).

VEGFRs are type-V RTKs composed of an extracellular domain, a transmembrane region and an intracellular tyrosine kinase domain (Shibuya et al., 1990, Terman et al., 1991, Matthews et al., 1991) (**Figure 1.**). Extracellular domains of VEGFRs consist of seven immunoglobulin (Ig) homology domains (Terman et al., 1991), where the second Ig domain is required for ligand binding promoting receptor dimerization and the third domain is needed to strengthen ligand-receptor interaction (Joukov et al., 1996, Leppanen et al., 2010).

VEGFR1 (Flt-1) was the first RTK of the VEGFR family to be identified (de Vries et al., 1992), but its specific function is still unclear, because VEGFR1 can signal differentially depending on cell type and developmental stage (Ferrara, 2004). Recently published data in mouse embryonic stem cell derived-vessels lacking *Vegfr1* demonstrated that vessel branching was significantly decreased whereas initiation of vessel sprout and connectivity with other vessels was increased (Chappell et al., 2016). VEGFR1 is a receptor for the VEGF, VEGF-B and PlGF ligands (de Vries et al., 1992, Park et al., 1994, Olofsson et al., 1998) and it is upregulated in hypoxia via HIF-1α (Gerber et al., 1997). VEGFR1 is able to undergo alternative splicing resulting in a soluble form of VEGFR1, which can inhibit VEGF activity and signalling (Kendall and Thomas, 1993). VEGFR1 is essential during embryonic development and VEGFR1 deletion leads to overgrowth of endothelial-like abnormal cells within vascular lumens and embryonic death between E8.5 and E9.5 (Fong et al., 1995, Fong et al., 1999). Interestingly, mice lacking the VEGFR1 tyrosine kinase domain ($Flt1^{TK-/-}$) can still bind VEGF, and have normal vascular development indicating that VEGFR1 inhibits VEGF activity (Hiratsuka et al., 1998).

VEGFR2 (kinase insert domain-containing receptor (KDR), human; fetal liver kinase 1 (Flk-1)), is the key regulator of VEGF induced EC proliferation and migration (reviewed in Roskoski, 2008). VEGF binds with lower affinity to VEGFR2 compared to VEGFR1, but stimulates stronger tyrosine kinase activity of VEGFR2 than VEGFR1 (Waltenberger et al., 1994). In addition to VEGFR2 expression in ECs, VEGFR2 expression has been found also in neuronal cells, retinal progenitor cells, megakaryocytes and hematopoietic stem cells (Katoh et al., 1995). VEGFR2 is essential for early embryonic vasculogenesis and blood-island formation, *Flk-1* deficient mice die between E8.5-E9.5 because of abrogation of hematopoietic and EC differentiation (Shalaby et al., 1995). VEGF binding to the second and third Ig-like domains of VEGFR2 leads to receptor dimerization and strong phosphorylation of intracellular tyrosine residues (Fuh et al., 1998). VEGF-induced VEGFR2 phosphorylation stimulates numerous downstream signalling pathways by phosphorylating phospholipase Cγ, ras GTPase activating protein, phosphoinositide 3-kinase (PI3K) and Scr family kinases (Guo et al., 1995, Eliceiri et al., 1999). In

addition, VEGF stimulated VEGFR2 activation leads to activation of $\alpha_v\beta_3$-, $\alpha_v\beta_5$-, $\alpha_5\beta_1$-, $\alpha_2\beta_1$-integrins, which are dependent on PI3K - a serine/threonine kinase (Akt) signalling axis (Byzova et al., 2000). VEGFR2 activation induces also Raf-Mek-Erk signalling pathway via protein kinase C (PKC) resulting in EC proliferation (Takahashi et al., 1999).

As mentioned above, VEGFR3 binds VEGF-C and VEGF-D ligands and it is a main regulator of lymphangiogenesis, and formation of lymphatic vasculature (Joukov et al., 1996, Achen et al., 1998, Tammela and Alitalo, 2010). VEGFR3 is expressed mainly in lymphatic ECs, but it is detected also in blood vascular ECs during embryonic development (Kaipainen et al., 1995). VEGFR3 can heterodimerize with VEGFR2 following VEGF-C stimulation inducing sprouting angiogenesis (Tammela et al., 2008, Nilsson et al., 2010). Although VEGFR3 is downregulated in adult blood vessels, it has been shown that VEGFR3 is upregulated in tumor angiogenesis (Valtola et al., 1999, Kubo et al., 2000). The activation of VEGFR3 induces downstream signalling pathways such as the PI3K-Akt pathway and phosphorylation of PKC-mediated p42/p45 mitogen activated protein kinase (MAPK) (Makinen et al., 2001).

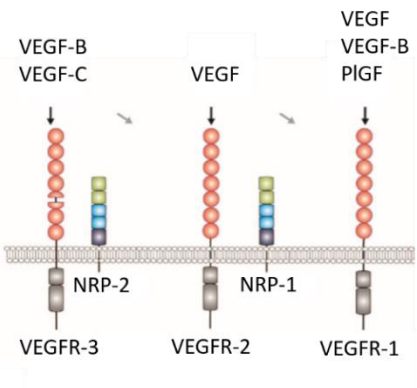

Figure 1. Vascular endothelial growth factors and their receptors and co-receptors. VEGF, Vascular endothelial growth factor; VEGFR, Vascular endothelial growth factor receptor; NRP, Neuropilin; PlGF, placental growth factor. The image is modified from (Saharinen et al., 2011).

1.2.2. Angiopoietin-Tie system

Besides the VEGF-VEGFR system, angiopoietin growth factors together with their EC specific tyrosine kinase receptors Tie1 and Tie2 regulate vascular morphogenesis during embryonic development (Augustin et al., 2009, Eklund and Saharinen, 2013). In the mature adult vasculature, the Ang-Tie system contributes to inflammation, regulates endothelial barrier function and inflammatory vascular remodelling, and participates in pathological angiogenesis and lymphangiogenesis, for example in cancer (Augustin et al., 2009, Eklund and Saharinen, 2013)

The Tie receptors are type 1 transmembrane protein RTKs (Ramsauer and D'Amore, 2002), and they are expressed mainly in ECs (Dumont et al., 1994). Tie2 is also expressed in a muscle satellite cells (Abou-Khalil et al., 2009), in hematopoietic stem cells (Iwama et al., 1993, Ito et al., 2016) and Tie2 expressing

monocytes/macrophages (TEMs) (De Palma et al., 2003, De Palma et al., 2005). The ectodomain of these receptors consists of three fibronectin type III repeats (FN III), three epidermal growth factor (EGF)-like repeats and three Ig -like domains (Barton et al., 2006) (**Figure 2.**).

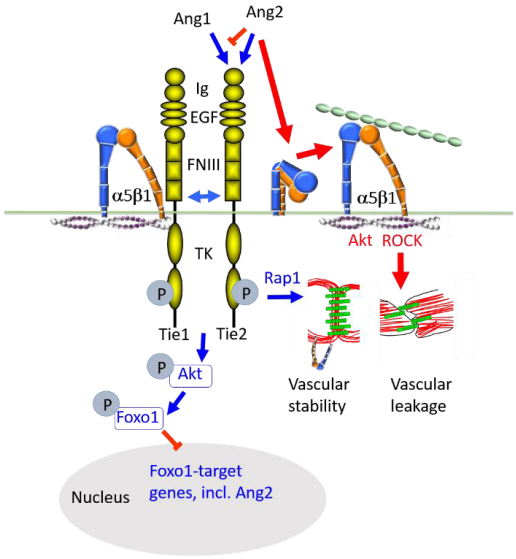

Figure 2. The structure of the Tie receptors and a summary of Ang-Tie signalling. Ang1, Angiopoietin 1; Ang2, Angiopoietin 2; Ig, immunoglobulin like domain; EG, epidermal growth factor like repeat; FN III, fibronectin type III repeats; TK, tyrosine kinase; Akt, a serine/threonine kinase; Foxo1, the Forkhead box protein O1; Rap1, Ras-proximate-1 or Ras-related protein 1; ROCK, Rho-associated protein kinase; $\alpha_5\beta_1$, $\alpha_5\beta_1$-integrin; P, phosphorylation.The image is modified from (Hakanpaa et al., 2015).

Angiopoietins bind to the Ig-EGF region of the Tie2 receptor (Fiedler et al., 2003, Barton et al., 2005), whereas no ligand has been identified for Tie1. Ligand binding to Tie2 leads to receptor complex formation with the Tie1 receptor (Saharinen et al., 2005). The intracellular region of Tie2 is composed of a tyrosine kinase domain and C-terminal tyrosine phosphorylation sites, which are phosphorylated after ligand binding (Davis et al., 1996, Shewchuk et al., 2000). During embryonic development, Tie2 is necessary for cardiovascular development after mid gestation, and *Tie2* deleted mouse embryos die between E10.5 to E12.5 due to the severe cardiac defects, lack of vascular remodelling and vessel maturation (Dumont et al., 1994, Sato et al., 1995).

The function of the orphan Tie1 receptor is not fully understood. Tie1 expression is induced by VEGF and hypoxia in activated ECs (McCarthy et al., 1998). In addition, disturbed blood flow in branched blood vessels induces Tie1 expression (Porat et al., 2004). *Tie1* deficient mouse models have shown significant biological functions for Tie1. *Tie1* null mouse embryos have impaired endothelial integrity and hemorrhages leading to lethality around E13.5 (Sato et al., 1995, Puri et al., 1995). In addition,

D'Amico et al. (2010) showed in another genetic background that Tie1 is also essential for lymphatic development; the *Tie1*$^{-/-}$ mouse embryos had malformed lymphatic vessels and edema, the embryos were swollen before signs of hemorrhage. Tie1 is also necessary for lymphatic valve formation and lymphatic remodelling and maturation (Shen et al., 2014, Qu et al., 2015). In addition, Woo and Baldvin (2011) consider that Tie1 could participate to atherosclerosis.

The angiopoietin family consists of Ang1, Ang2 and Ang4 (the human orthologue of mouse Ang3) growth factors (also termed Angpt1, Angpt2 and Angpt4) (Davis et al., 1996, Maisonpierre et al., 1997, Valenzuela et al., 1999, Lee et al., 2004). The angiopoietin structure consists of an N-terminal super-clustering domain (SCD) followed by a central coiled-coin domain (CCD), and C-terminal fibrinogen-like domain (Davis et al., 1996, Maisonpierre et al., 1997, Valenzuela et al., 1999). The CCDs of angiopoietins enable their homo-oligomerization, and the SCD further clusters angiopoietins into larger oligomers, including tetramers, and Ang1 in addition to larger multimers (Kim et al., 2005b). Angiopoietins bind Tie2 via their fibrinogen-like domain (Procopio et al., 1999).

Ang1 is a strong Tie2 agonist and it also activates Tie1 (Saharinen et al., 2005). Ang1 stimulates the translocation of Tie receptors into EC-EC contacts resulting in EC stabilization (Fukuhara et al., 2008, Saharinen et al., 2008). In mice, Ang1 promotes structural integrity, survival and maturation of newly formed blood vessels, likely via its activity in EC-EC junctions (reviewed in Brindle et al., 2006). Tie2 promotes cell survival via activation of the PI3K-Akt serine kinase pathway resulting in activation of the endothelial nitric oxic synthetase (eNOS) (Kim et al., 2000). Activation of the Akt pathway results also to nuclear exclusion and inhibition of the Forkhead box protein O1 (Foxo1) transcription factor (Daly et al., 2004) (**Figure 2**.). Foxo1 inhibition leads to downregulation of target genes involved in endothelial destabilization, metabolism, growth control and apoptosis (Brunet et al., 1999, Kim et al., 2000, Zhang et al., 2002, Wilhelm et al., 2016). Signalling in mobile EC is organized differentially: Tie2 is activated in EC-ECM adhesions via matrix-bound Ang1. Tie2 activation in mobile cell fosters activation of Erk and DokR, cell migration and matrix adhesion (Fukuhara et al., 2008, Saharinen et al., 2008).

During early embryonic development (E9-E11), Ang1 is expressed in the embryonic heart myocardium surrounding the endocardium and it is essential for embryonic vascular development: *Ang1* deficient mouse embryos die at the embryonic day E12.5. Even when the vasculature has developed, *Ang1* deficient mice have less small vessels because of decreased branching (Suri et al., 1996). Instead, later during development Ang1 is expressed in mesenchymal cells surrounding the developing vessels (Davis et al., 1996). Ang1 forms tri-, tetra- and pentameric homo-oligomers that can further cluster into multimeric structures via its amino-terminal SCD. In order to activate Tie2 in ECs, Ang1 must exist at least in a tetramer structure (Davis et al., 2003, Kim et al., 2005b), although recently published data demonstrate that specifically designed recombinant dimeric Ang1 forms can activate Tie2 (Oh et al., 2015). Embryonic deletion of Tie2 results in lethality at around E10.5 with very similar embryonic

phenotype as observed in Ang1 null embryos (Dumont et al., 1994, Suri et al., 1996). However, Ang1 is dispensable in adult mice under baseline conditions, but required to attenuate aberrant pathological responses, such as in the diabetic kidney (Jeansson et al., 2011).

Ang2 may act as a weak agonist or antagonist of Tie2 depending on the context (Maisonpierre et al., 1997). Ang2 is expressed by ECs where it is stored in special secretory granules, termed Weibel-Palade bodies (Fiedler et al., 2004). In adults, Ang2 expression is low in the quiescent vasculature, but elevated in several diseases, such as sepsis (Parikh et al., 2006), atherosclerosis (Post et al., 2008), diabetes (Lim et al., 2004) and in many cancers including breast cancer, melanoma, colorectal cancer, RCC and glioblastoma (Sfiligoi et al., 2003, Helfrich et al., 2009, Goede et al., 2010, Wang et al., 2014, Scholz et al., 2016). Upregulation of Ang2 expression in tumors promotes tumor growth, vascular de-stabilization and metastasis, which can be prevented using Ang2 blocking Abs (Holash et al., 1999, Holopainen et al., 2012, Leow et al., 2012). In addition, Ang2 expression increases in the tumor vasculature in response to hypoxia (Oh et al., 1999).

During development, Ang2 agonist activity is required for the development of lymphatic vasculature (Gale et al., 2002), whereas Ang2 overexpression in transgenic mouse embryos leads to embryonic death during E9.5-E10.5 with vascular disorder, a similar phenotype that is detected in *Ang1* or *Tie2* deleted mouse embryos (Maisonpierre et al., 1997). Double deletion of Ang1 and Ang2 leads to development of glaucoma, and loss of function *Tie2* mutations in humans cause primary congenital glaucoma (Thomson et al., 2014, Souma et al., 2016). In contrast, hyperactivated Tie2 mutants result in venous malformations in human patients (Boscolo et al., 2015).

1.2.3. Regulation of Ang-Tie signalling by Tie1 and integrins

The Tie receptors undergo a unique cellular microenvironment-dependent mechanism of activation, stimulated by angiopoietins, leading to Tie receptor accumulation in the EC-EC junctions of endothelial monolayers. Instead, Tie receptors are accumulated on ECM contacts of the rear of the moving ECs (Fukuhara et al., 2008, Saharinen et al., 2008).

Several studies show that the extracellular domain of Tie2 can undergo cleavage, which is induced by VEGF or phorbol myristate acetate (PMA) (Reusch et al., 2001, Findley et al., 2007, Onimaru et al., 2010). Soluble Tie2 (sTie2) has been detected in normal healthy human serum, and increased levels in coronary artery disease, congestive heart failure and RCC (Harris et al., 2001, Chung et al., 2003, Chong et al., 2004). Also, cleavage of the extracellular domain of Tie1 by metalloprotease activity has been reported (McCarthy et al., 1999, Yabkowitz et al., 1999). Soluble Tie1 (sTie1) is detected in normal blood circulation, but sTie1 levels are elevated by PMA, VEGF, in inflammatory conditions by tumor necrosis factor-α (TNF-α) and shear stress in culture conditions (McCarthy et al., 1999, Yabkowitz et al., 1999, Chen-Konak et al., 2003). Elevated levels of sTie1 are detected in lung, ovarian and breast cancer patients with thoracic or pelvic metastasis (Karnani and Kairemo, 2003). In

contrast to Tie2 ectodomain, Tie1 is unable to bind Ang1, and the cleaved sTie1 in circulation is not able to inhibit Ang1 induced Tie receptor signalling. Marron et al. (2007) demonstrated that cartilage oligomeric matrix protein (Comp) -Ang1 induced stronger Tie2 phosphorylation after Tie1 cleavage in ECs and suggested that the ability of Tie2 to bind ligand was increased after Tie1 cleavage. A similar effect was detected in *Tie1* silenced cells. Instead, recently published data demonstrate that Tie1 cleavage in inflammation promotes the Tie2 antagonist activity of Ang2 suggesting that Tie1 is necessary for Ang-Tie signalling (Korhonen et al., 2016).

Integrins regulate EC-ECM adhesion, and they also participate in angiogenesis (Hodivala-Dilke et al., 2003). Several studies have suggested that angiopoietins can interact with integrins (Cascone et al., 2005, Hakanpaa et al., 2015). Ang2 can bind to $\alpha_v\beta_3$-, $\alpha_v\beta_5$-, and $\alpha_5\beta_1$-integrins, but interacts most strongly with $\alpha_5\beta_1$-integrin (Felcht et al., 2012, Lee et al., 2014). Chen et al. (2009) demonstrated that Ang1 induced neurite growth occurred via the β_1-integrin and activated focal adhesion kinase (FAK)-PI3K-Akt pathway in Tie2 negative PC12 cell line. In addition, Lee et al. (2013) demonstrated that Ang1 induce EC migration and activate FAK via $\alpha_v\beta_5$-integrin in *Tie2* negative retinal astrocytes. Carlson et al. (2001) compared the effect of Ang1 on cell adhesion and sprouting using ECs and Tie2 negative fibroblasts. Similar results were seen in these different cell lines suggesting that Ang1 induced cell adhesion and sprouting occur independently of Tie2. Interestingly, Ang1 mediated cell adhesion was inhibited with integrin blocking Abs (Carlson et al., 2001). Recently published data demonstrate that Ang2 activates β_1-integrin leading to endothelial destabilization, but this effect was not seen with Ang1 (Hakanpaa et al., 2015).

1.3. Formation of the blood vascular system

1.3.1. Molecular regulation of angiogenesis

The growth of new blood vessels is a highly controlled process regulated by angiogenic factors. The formation of blood vasculature can be classified into two main forms: vasculogenesis and angiogenesis. Vasculogenesis during early embryonic development is initiated by the formation of blood islands from the mesoderm layer of the yolk sac. The blood islands are clusters of hemangioblasts that differentiate to hematopoietic precursor cells and endothelial progenitor cells (reviewed in Ferguson et al., 2005). These EC progenitors then coalesce to form the primitive vascular plexus. Angiogenesis, the formation of the new blood vessels from pre-existing blood vessels, occurs during later stages of embryonic development and also throughout the adulthood (Ferguson et al., 2005). In adult organisms, physiological angiogenesis takes place for example during wound healing and tissue regeneration (Tonnesen et al., 2000), menstrual cycle (Augustin, 2005), and exercise-induced skeletal muscle growth (Prior et al., 2004). Angiogenesis is also a key process of many pathological conditions, including tumor angiogenesis promoting cancer progression, rheumatic disease and neovascular eye diseases, such as wet age-related macular degeneration (wAMD) (Risau, 1997, Ambati and Fowler, 2012). The formation of new blood vessels is regulated by two main RTK families: VEGFs and their receptors, and

angiopoietins and their Tie receptors in co-operation with other cellular pathways, including the Notch pathway (Jeltsch et al., 2013).

1.3.2. Tip and stalk cells

A highly investigated form of angiogenesis is sprouting angiogenesis, where specialized ECs are required to initiate and lead the growth of a new vessel branch. In the front line of sprouting vessels are specialized tip cells, which extend filopodia to probe and invade the microenvironment in response to VEGF gradient (**Figure 3.**) (Gerhardt et al., 2003). VEGF is secreted by hypoxic cells and is bound to the cell matrix, thereby providing guidance for tip cell migration. Behind the tip cells, stalk cells ensure the elongation of the stalk of the sprout by proliferation and forming the lumen (De Smet et al., 2009). Tip cells proliferate minimally whereas stalk cells proliferate forming EC-EC junctions to stabilize the nascent vessel (Carmeliet and Tessier-Lavigne, 2005). The most quiescent ECs are termed as phalanx cells, which are covered by perivascular cells, either pericytes or vascular SCMs, and tightly adherent to each other forming tight EC junctions regulating perfusion of newly formed blood vessels (De Smet et al., 2009).

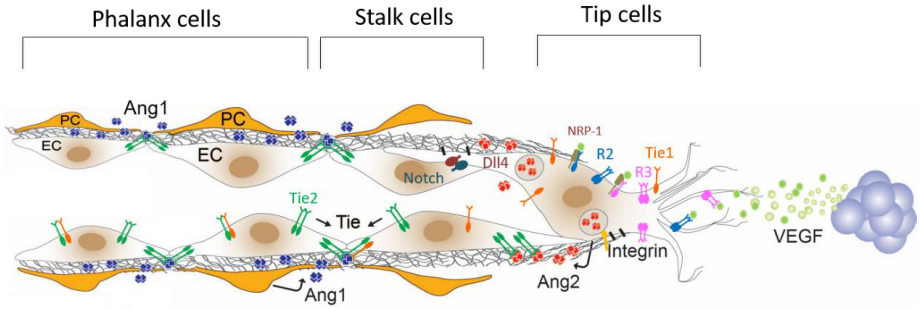

Figure 3. Receptors in sprouting angiogenesis. VEGFR2, VEGFR3, NRP-1, Dll4 and Tie1 receptors are expressed in tip cells. Tie2 is expressed in stalk cells. VEGF, Vascular endothelial growth factor; R2/3, Vascular endothelial growth factor receptor 2/3; NRP-1, Neuropilin-1; PC, Pericyte; EC, Endothelial cell; Dll4, Delta-like ligand-4; Notch, Notch receptor. The image is modified from (Jeltsch et al., 2013).

Tip cells have specific molecular signalling components to regulate their specification as a tip cell. Tip cells express for example VEGFR2, VEGFR3, Delta-like ligand-4 (Dll4) and NRP-1 and NRP-2 (Gerhardt et al., 2003, Gerhardt et al., 2004, Suchting et al., 2007, Tammela et al., 2008). NRPs are co-receptors for VEGFR2 and VEGFR3 and NRP-1 is relevant for the function of tip cells (Favier et al., 2006, Pan et al., 2007). Also, Ang2 is highly expressed in tip cells (Felcht et al., 2012).

In addition to vessel invasion to the surrounding tissue, the angiogenic process requires the degradation of ECM. ECM regulates the expression of matrix metalloproteinases (MMPs) that is expressed by tip cells to enhance the new forming vessel invasion (De Smet et al., 2009). Tip cells express membrane type-1 MMP

(MMP14), which plays a key role during angiogenesis and is downregulated in the stalk cells of stabilized vessels (Yana et al., 2007, van Hinsbergh and Koolwijk, 2008).

VEGF binding to VEGFR2 induces several signalling pathways leading to EC specification as tip cells, including the formation of filopodia and highly polarized phenotype, and resulting in the initiation of migration towards the angiogenic stimulus (Gerhardt et al., 2003, De Smet et al., 2009). During this process, VEGF induced Dll4 expression leads to activation of the Notch pathway in adjacent ECs resulting in the inhibition of VEGFR2 activity in the stalk cells (Roca and Adams, 2007). Tie2 is expressed in stalk and phalanx cells, but its expression is low in tip cells (del Toro et al., 2010). This may be due to Tie1 expression in tip cells resulting in inhibition of Tie2 presentation on the cell surface (Marron et al., 2007, Savant et al., 2015).

Activation of the Notch signalling pathway in stalk cells leads to inhibition of vessel branching, decreasing filopodia expression and inhibition of the migratory response to VEGF via downregulation of VEGFR2 and VEGFR3 (Tammela et al., 2008, Williams et al., 2008, Jakobsson et al., 2009). For example in tumor models, inhibition of the Notch signalling pathway using anti-Dll4 Abs, or viral vectors encoding a soluble dimerized version of the extracellular region of Dll4 fused to the human IgG1 Fc constant region, leads to stalk cell differentiation into tip cells, tip cell migration and vessel branching and increased vessel density, but interestingly, decreased tumor growth due to non-functional vasculature (Noguera-Troise et al., 2006, Ridgway et al., 2006). The postnatal retina is a highly used model to investigate sprouting angiogenesis, and many of the above mentioned results are observed in the retina (Gerhardt et al., 2003, Dorrell and Friedlander, 2006). Retinal vascular development is impaired in conditional deletions of Ang1, Ang2 and Tie1, indicating the requirement of the Ang-Tie pathway in physiological postnatal sprouting angiogenesis (Jeansson et al., 2011, Lee et al., 2013, D'Amico et al., 2010).

1.4. Tumor angiogenesis and features of the tumor vasculature

Nutrients and oxygen are essential for the growth and enlargement of tumors, and tumors also need to remove carbon dioxide and other metabolic wastes, similarly to normal tissues. All these functions require a functional blood circulation, created by tumor-associated neo-vascularization. In adult, the normal vasculature is quiescent for the majority of time, but during tumor progression “the angiogenic switch” will turn a program that results in new vessel growth from nearby vessels. This switch occurs, via activation of a variety signalling mechanisms, most importantly VEGF and angiopoietin signalling pathways (Hanahan and Weinberg, 2011). Similar to the normal vasculature, the tumor vasculature consists of ECs, mural cells (pericytes or vascular SMCs) and the BM, but all of these constituents are abnormal and their functions altered from the normal vasculature. One example is the altered VEGF dependence of the tumor endothelium, sustaining angiogenesis even in the presence of VEGF blocking therapy and creating a challenge for development of effective anti-angiogenic cancer therapies (Baluk et al., 2005). In contrast to the vasculature in healthy tissues, proangiogenic signals induced in the tumor stimulate functional and

structural abnormalities in tumor vessels; the blood flow is erratic, proliferation and apoptosis of ECs is abnormal creating a poorly organized vascular network, vessels undergo sprouting, remodelling, and proliferation in response to constant VEGF signalling, and vessel enlargement and branching occurs (Baluk et al., 2005, Nagy et al., 2010). In contrast to healthy vasculature, tumor vasculature does not contain a normal tight EC layer and tight EC-EC junctions, which leads to vessel leakiness contributing to tumor metastasis. Some tumors are especially bloody with large "blood lakes", and tumor hemorrhage is an indication of abnormal and leaky vasculature with a defective endothelial barrier function (Hashizume et al., 2000). In addition to high VEGF expression, which stimulates vessel leakiness, another reason for leaky tumor blood vessels can be the decreased attachment of pericytes to ECs (Abramsson et al., 2002, Morikawa et al., 2002). Several studies have shown that alterations on plateled-derived growth factor (PDGF) signalling affect the association of pericytes to tumor vessels (Abramsson et al., 2002, Abramsson et al., 2003, Furuhashi et al., 2004). In addition, Holash et al. (1999) describe that in a rat glioma model, pericyte detachment and regression was associated with Ang2 expression in ECs leading to tumor hypoxia, upregulation of VEGF expression and tumor angiogenesis.

Vascular BM with MMPs and vascular integrins regulate tumor angiogenesis. Also, the tumor vascular BM differs from normal tissues. The vascular BM of normal tissue vasculature consists of type III, IV and VII collagen, laminin, nidogens, and proteoglycans forming a tight envelope between the ECs and pericytes (reviewed in Kalluri, 2003). Instead, in tumors, BM is not associated with ECs and consists of focal holes, and has an altered composition including for example upregulated expression of fibronectin and its receptor $\alpha_5\beta_1$-integrin (reviewed in Baluk et al., 2005).

1.4.1. VEGFs and Ang2 in tumor angiogenesis

The VEGF-VEGR2 signalling axis is the major regulator of tumor angiogenesis, and many tumors including neoplastic tumor and stromal cells, secrete VEGF (Ferrara, 2004, Ferrara and Adamis, 2016). RCC is an example of a tumor, which expresses VEGF mRNA at a very high level (Tomisawa et al., 1999, reviewed in Ferrara et al., 2007). VEGF is also detected by immunohistochemical (IHC) staining in breast (Yoshiji et al., 1996) and kidney cancer (Nicol et al., 1997), and elevated serum VEGF levels are detected in ovarian cancer (Yamamoto et al., 1997). VEGF mRNA is elevated also in pituitary adenomas (Lloyd et al., 1999) and thyroid cancer (Soh et al., 1997). VEGF is upregulated by hypoxia and it is a target gene of the HIF transcription factor. HIF stability is regulated by von Hippel-Lindau (VHL) tumor suppressor that in normoxic conditions targets HIF to degradation (Safran and Kaelin, 2003).

The importance of VEGF to tumor angiogenesis and tumor growth was originally shown by Kim et al. (1993), who demonstrated in pioneering studies that a monoclonal Ab against VEGF decreased the growth of tumors, and the density of tumor vessels in mice. Subsequently, tumor growth inhibition was demonstrated in many other studies using various VEGF blocking agents. Millauer et al. (1994) showed that the growth of human glioblastoma tumor xenografts was inhibited in nude mice expressing

a dominant-negative mutant of the Flk-1/VEGF receptor via viral vector delivery. Also, adenoviral mediated gene transfer of soluble VEGF receptor (or a fusion of the first three Ig domains of VEGFR1 and the constant region of human IgG1) inhibited tumor growth in immunodeficient mouse tumor models (Goldman et al., 1998, Kong et al., 1998, Holash et al., 2002). Prewett et al. (1999) demonstrated that Ab against VEGF receptor flk-1 (VEGFR2) decreased MVD and tumor cell proliferation, and tumor necrosis was extensive in experimental mouse tumors. Willet et al. (2004) first demonstrated that blocking VEGF has clinical benefit in human patients, by showing that blocking human VEGF with a humanized monoclonal Ab (bevacizumab) in cancer patients decreases tumor vascular volume and microvascular density, tumor perfusion and interstitial fluid pressure. The humanized anti-VEGF Ab bevacizumab inhibits VEGF-VEGFR2 interaction, and was the first anti-angiogenic therapy accepted in clinical use, for the treatment of metastatic colorectal cancer (Ferrara and Kerbel, 2005, Strickler and Hurwitz, 2012). Since then, additional VEGF-VEGFR2 targeted therapies have been developed and approved for use in a wide variety of human cancers. Despite of clear tumor control benefit in certain cancers, not all tumors are sensitive to VEGF-targeted therapies and those that initially respond, often become resistant to the treatment (Abdullah and Perez-Soler, 2012, Sennino and McDonald, 2012, Singh and Ferrara, 2012). One potential explanation for therapy associated resistance is that tumors may be able to switch and use another receptor-ligand pathway instead of the VEGF pathway and this way maintain vessel sprouting and tumor angiogenesis in the presence of VEGF inhibitors. For example, the tumor vasculature expresses also VEGFR3 which can be activated by VEGF-C and VEGF-D, produced by tumor and inflammatory cells, or in addition, the proteolytically processed forms of VEGF-C and VEGF-D can activate VEGFR2 directly (Joukov et al., 1997, Stacker et al., 2001, Karpanen and Alitalo, 2008).

Besides the VEGF family and their receptors, also the Ang-Tie system is involved in tumor angiogenesis. High circulating Ang2 levels and increased mRNA expression have been found to predict for poor prognosis in melanoma (Helfrich et al., 2009), metastatic colorectal cancer (Goede et al., 2010), glioblastoma (Scholz et al., 2016) and in breast cancer (Sfiligoi et al., 2003). ECs of RCC and human glioblastoma have been reported to express Ang2 (Rautiola et al., 2016, Scholz et al., 2016).

Blocking Ang2 in mouse tumors reduces tumor angiogenesis by reducing the number of angiogenic sprouts, and the combination of Ang2 and VEGF blocking inhibitors shows increased efficacy when compared to either monotherapy (Brown et al., 2010). Oliner et al. (2004) showed that using anti-Ang2 Abs and peptide-Fc fusion proteins that prevent the interaction of Ang2 and Tie2 receptor resulted in reduced EC proliferation and inhibition of tumor growth in human epidermoid and colorectal tumor xenografts. Importantly, we showed that blocking Ang2 inhibits significantly tumor growth by reducing the number of tumor blood vessels in lung cancer xenografts. Blocking Ang2 also reduced the number of lymphatic vessels and lymph node metastasis, as well as metastasis to lungs by improving EC junctions of metastasis associated capillaries (Holopainen et al., 2012). Interestingly, several studies show that anti-Ang2 treatment combined to VEGF blocking inhibited tumor

growth in mouse models (Hashizume et al., 2010, Daly et al., 2013, Kienast et al., 2013). Falcón et al. (2009) demonstrated that a peptibody Ang2 inhibitor (L1-7[N]) reduced tumor growth and decreased the number of blood vessels in human colon xenografts. Ang2 blocking treatment resulted in tumor vascular normalization including accumulation of vascular endothelial cadherin (VE-cadherin) to the EC-EC junctions; increased pericyte coverage around the vessels and reduced endothelial sprouting. Pericytes are an important element to stabilize tumor vessels. Keskin et al. (2015) showed that targeting pericytes resulted in elevated Ang2 levels in a human mammary tumor xenograft mouse model leading to leaky vessels and increased lung metastasis, but the combination with blocking Ang2 treatment restored vascular stabilization, decreased metastasis and supressed tumor growth.

Ang1 overexpression in human xenograft models results in pericyte covering of the blood vessels (Hawighorst et al., 2002). Interestingly, mixed effects on tumor growth have been demonstrated by endogenous or exogenous viral vector-mediated Ang1 overexpressing in certain tumor models. Ang1 has been shown to inhibit tumor growth in several studies (Hayes et al., 2000, Hawighorst et al., 2002, Stoeltzing et al., 2003), but tumor growth promoting effects have been also indicated (Machein et al., 2004). Recombinant Ang1 has been demonstrated to stimulate EC proliferation resulting in vascular enlargement in mouse post-natal retina and postcapillary venules indicating that Ang1 participates and stimulates vessel remodelling in normal tissue (Thurston et al., 2005, Lee et al., 2013). In tumors, blocking Ang1 had almost no effect on the vasculature, instead, together with an Ang2 inhibitor, Ang1 inhibitor prevented tumor vessel normalization indicating that Ang1 has a stabilizing effect on the vasculature (Falcon et al., 2009).

Tie1 expression is increased in human and mouse tumor vessel, and *Tie1* deletion in the endothelium of adult mice resulted in inhibition of tumor growth and angiogenesis (Kaipainen et al., 1994, D'Amico et al., 2014). Tumors grown in *Tie1* deficient mice had less tumor blood vessels and less tumor cell proliferation when compared to control, wild type (WT) mice (D'Amico et al., 2014). *Tie1* deletion also increased apoptosis in ECs and tumor cells, and decreased vessel sprouts.

1.5. Regulation of vascular permeability

1.5.1. Endothelial cell-cell junctions

Cellular junctions are important for EC communication and adhesion to the surrounding cells and tissues. The junctional EC transmembrane proteins are anchored to the actin cytoskeleton via specific intracellular proteins to stabilize the junctions and transfer the intracellular signals (reviewed in Bazzoni and Dejana, 2004). Also, the dynamic regulation of junction opening and closing is provided by transmembrane receptor adhesion to the cytoskeleton (Hartsock and Nelson, 2008). The EC junctions have been categorized as adherent, tight and gap junctions and are formed according to vessel type and permeability requirements of the target organ. Whereas the gap junctions provide the communication between the neighbouring cells, tight junctions enable the “barrier” by regulating the vessel permeability, and they also sustain cell

polarity. Maturation and maintenance of EC-EC contacts are provided by adherent junctions (Bazzoni and Dejana, 2004, Dejana et al., 2009). Both, the adherent and the tight junctions participate in the transfer of the intracellular signals that control many EC functions (Dejana et al., 2009). In general, junctions are functional structures that change according to their environment, and also intracellular adhesive proteins that are attached to the transmembrane proteins vary during the junction maturation and stabilization (Ayalon et al., 1994, reviewed in Bazzoni and Dejana, 2004) (**Figure 4.**).

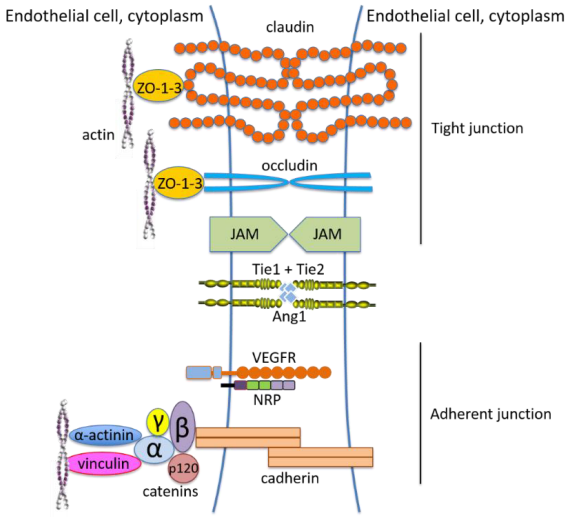

Figure 4. Tight and adherent junctions in endothelial cells. ZO-1-3, Zonula occludens 1-3; JAM, junction adhesion molecule; Ang1, Angiopoietin-1; VEGFR, Vascular endothelial growth factor receptor; NRP, Neuropilin; α/β/γ/p120, Catenins. The image is modified from (Dejana, 2004).

Cell adhesion at adherent junctions is mediated by proteins of the cadherin family, including N-cadherin and VE-cadherin that are expressed in all vessel types. Both cadherins bind by their cytoplasmic C-terminal domains to catenins, such as β-catenin, p120 and plakoglobin. β-catenin and plakoglobin bind to the actin binding protein α-catenin (Vestweber, 2008). α-catenin binds to the actin-binding proteins vinculin (Weiss et al., 1998), α-actinin (Knudsen et al., 1995) and zonula occludens-1 (ZO-1), which is mainly present at tight junctions (Itoh et al., 1997). This catenin-actin dependent mechanism has been identified to strengthen the EC junctions, but interestingly, Yamada et al. (2005) challenged this theory by showing that actin was unable to bind to the E-cadherin-β-catenin-α-catenin complex. Still, catenin deficiency destabilized the cadherin mediated cell adhesion (Vestweber, 2008).

VE-cadherin is essential for regulating vascular permeability and integrity. Corada et al. (1999) demonstrated that administration of the anti-VE-cadherin Abs increased vascular permeability, fragility and hemorrhages *in vivo*. The same report demonstrated in cultured cells, that the Ab blocking of VE-cadherin induced redistribution of VE-cadherin, but it did not affect the tight junction marker ZO-1.

VEGFR2 has been shown to associate with VE-cadherin and this association is rapidly dissociated after VEGF stimulation (Weis et al., 2004). In addition to stimulation of angiogenesis, VEGF is also a powerful factor inducing vessel leakiness. VEGF activation of VEGFR2 signalling induces the tyrosine phosphorylation of VE-cadherin, β-catenin, γ-catenin/plakoglobin and p120-catenin resulting in dismantling of EC-EC contacts (Esser et al., 1998). Additional mechanisms have been reported that contribute to VEGF-induced vascular leakiness. Wu et al. (2003) showed that inhibition of FAK that is induced by VEGF and participate in EC adhesion and migration, significantly decreased VEGF elicited vascular hyperpermeability *in vivo*. Wu et al. (1999) showed that inhibition of eNOS, PLC-ϒ, or PKC blocked VEGF induced vascular permeability *in vivo*. Phosphorylation of VE-cadherin may be also due to inhibition of associated phosphatases, including the receptor-type tyrosine-protein phosphatase beta (VE-PTP), which is reported to associate with VE-cadherin (Nawroth et al., 2002) and Tie2 (Fachinger et al., 1999). Baumer et al. (2006) demonstrated that homozygous VE-PTP mutant mouse embryos lacking the VE-PTP phosphatase domain, died at E10 without hierarchically organized vascular network and defects on heart development, and the growth of embryos between E8.5-E9.5 was significantly retarded. These results are in line with VE-cadherin deficient mice (Carmeliet et al., 1999) suggesting that vessel development is retarded in situation where VE-cadherin is constitutively phosphorylated. In addition, it has been reported that cleavage of VE-cadherin by metalloproteases can also promote vascular permeability (Herren et al., 1998).

As described above, VE-PTP has been reported to associate with Tie2, resulting in dephosphorylation of Tie2 (Fachinger et al., 1999). Treatment of ECs with a function blocking anti-VE-PTP Ab resulted in Tie2 phosphorylation and downstream activation of Erk1/2, which induced EC proliferation and vascular enlargement (Winderlich et al., 2009). Interestingly, in a recent study, Frye et al. (2015) demonstrated, that VE-PTP inhibition stabilized EC junctions via Tie2 *in vivo.* Blocking VE-PTP with a specific pharmacological small molecule inhibitor (AKB-9778) inhibited vascular leakiness induced by inflammatory mediators, and decreased leukocyte transmigration through the endothelial barrier. This was mediated by Tie2 induced Ras-proximate-1 (Rap1)-Rac1 signalling pathway resulting in the stabilization of cortical actin in ECs. Instead, in the absence of Tie2, VE-PTP blocking Ab destabilized endothelial junctions, which did not occur in the absence of VE-cadherin (Frye et al., 2015). Ang1 stimulation induces VE-PTP accumulation in EC-EC junction (Saharinen et al., 2008), where it also associates with Tie2, and dephosphorylates VEGFR2 (Mellberg et al., 2009). Thus, VE-PTP accumulation in EC-EC junctions in response to Ang1, may downregulate VEGFR2 signalling pathway promoting the barrier function (Mellberg et al., 2009).

Various growth factors, such as VEGF, as well as inflammatory mediator histamine and thrombin, and cytokines promote vascular leakiness, which can be inhibited by Ang1 (Thurston et al., 2000, Baffert et al., 2006). Several *in vitro* studies in ECs suggest that Ang1 can stabilize EC-EC junctions, decreasing EC monolayer permeability and modulating the activation of junctional molecules (Gamble et al.,

2000, Wang et al., 2004). Ang1 has been reported to promote the accumulation of the platelet endothelial cell adhesion molecule-1 (PECAM-1) to EC junctions and decrease the phosphorylation of VE-cadherin and PECAM-1 (Gamble et al., 2000). In contrast, Ang2 in concert with inflammatory cytokines has been reported to induce vascular leakiness (Benest et al., 2013).

Tight junctions are composed of transmembrane proteins such as occludins, claudins, junction adhesion molecules (JAMs), and intracellular proteins of the ZO family (reviewed in Bazzoni and Dejana, 2004). Occludin binds ZO-1 (Furuse et al., 1994), ZO-2 (Itoh et al., 1999b) and ZO-3 (Haskins et al., 1998). EC-EC adhesion in tight junctions is mediated mainly via the claudin family containing more than 20 members, of which only few, such as claudin-1, -3 and -5, are expressed in ECs (reviewed in Dejana et al., 2009). All claudins from claudin-1 to claudin-8 bind ZO proteins 1-3 (Itoh et al., 1999a). ZO family members are reported to bind to each other and associate with the cortical actin cytoskeleton, co-precipitating with F-actin. ZO-1 (Itoh et al., 1997) and ZO-2 (Itoh et al., 1999b) bind α-catenin (reviewed in Bazzoni and Dejana, 2004). Also, JAM family members and endothelial cell-selective adhesion molecule (ESAM) transmembrane glycoproteins are expressed in the tight junctions (reviewed in Dejana et al., 2009).

1.5.2. Inflammation

Vascular permeability and leukocyte transmigration are increased in inflammation. This phenomenon is associated with VE-PTP dissociation from VE-cadherin leading to phosphorylation of cytoplasmic residues of VE-cadherin resulting in leaky vessels (Broermann et al., 2011, Vockel and Vestweber, 2013, Wessel et al., 2014). VE-PTP is a target gene of HIF-2α, which is highly expressed in ECs and induced by hypoxia (Gong et al., 2015). Gong et al. (2015) demonstrated that HIF prolyl 4-hydroxylase 2 (PHD2) inhibitor stabilizes HIF-2α resulting in increased VE-PTP expression and dephosphorylation of VE-cadherin, which promoted the integrity of adherent junctions and endothelial barrier function in a lipopolysaccharide (LPS) induced inflammation model. In addition, VE-cadherin internalization is increased by inflammatory mediators (Gong et al., 2014), and VE-cadherin endocytosis leads to disassembly of adherent junctions and increases vascular permeability (Gavard and Gutkind, 2006, Gong et al., 2014).

As described above, Ang1 can inhibit vascular permeability via Tie2 (Thurston et al., 2000), whereas Ang2 has been described to be critical for cytokine-induced vascular leakage (Benest et al., 2013). Fuxe et al. (2011) demonstrated that vascular leakiness was prevented with Comp-Ang1 in *Mycoplasma Pulmonis* infected mice, and this required PDGF-dependent recruitment of pericytes. Basal and LPS-induced vascular permeability was increased after Tie2 silencing *in vivo* using siRNA in *Tie2* deficient mice, and reduced Tie receptor expression in mice predisposed to hemorrhagic complications of murine ebola hemorrhage fever, resulting resistance to a vascular leakiness indicating the importance of Tie2 for vascular barrier function (Rasmussen et al., 2014, Frye et al., 2015).

Increased Ang2 levels have been reported in sepsis (Parikh et al., 2006), which may due to decreased signalling via the Ang-Tie-Akt pathway resulting in Foxo-1 nuclear translocation and stimulation of Ang2 expression (Daly et al., 2006, Ghosh et al., 2015). For example, Ang2 was elevated and Tie2 phosphorylation was decreased in mucosal blood vessels in mice airways infected by *Mycoplasma Pulmonis* (Tabruyn et al., 2010). In this study, reduced Tie2 phosphorylation was supressed and remodelling of mucosal capillaries into venules was decreased when Ang2 blocking Abs were used to treat the mice (Tabruyn et al., 2010). In addition, the amount of leukocyte influx was decreased. Le et al. (2015) demonstrated that Ang2 blocking Abs inhibited vascular leakage and vessel enlargement synergistically, with TNF-α blocking Abs. TNF-α is an inflammatory cytokine upregulated in inflammation and sepsis (Tisoncik et al., 2012). Fiedler et al. (2006) showed in *Ang2* deficient mice that TNF-α induced leukocyte rolling on the luminal EC surface of the blood vessels but that leukocytes did not adhere to the activated endothelium. In addition, the same study showed that Ang2 sensitized ECs for TNF-α mediated upregulation of intercellular adhesion protein (ICAM-1). Interestingly, Han et al. (2016) showed recently that a novel Ang2 Ab ABTAA, that mediates clustering of ABTAA/Ang2-Tie2 complexes induced Tie2 phosphorylation. This Ab was more effective in vascular protection in sepsis compared to a conventional Ang2 blocking Ab, which inhibits Ang2 binding to Tie2.

It has been shown that Ang2 also binds to and signals via β_1-integrin (Felcht et al., 2012, Hakanpaa et al., 2015). Ang2 binding to β_1-integrin leads to actin stress fiber formation and changes in EC-ECM adhesion resulting in destabilization of the EC monolayer (Hakanpaa et al., 2015). Felcht et al. (2012) showed that Ang2 bound to abundantly expressed integrins in Tie2 deficient, angiogenically active ECs, resulting in phosphorylation of FAK and RAC-1 and leading to EC migration and sprouting angiogenesis. In addition, $\alpha_5\beta_1$-integrin has been demonstrated to co-immunoprecipitate (co-IP) with Ang2 in TNF-α stimulated ECs (Felcht et al., 2012). Tie2 expression has been described to decline in infections associated to leaky vessels, such as in sepsis, malaria, influenza and anthrax (reviewed in Ghosh et al., 2016).

2. Renal cell carcinoma (RCC)

2.1. Epidemiology and pathology

RCC is a highly vascular tumor, which accounts for approximately 2-3 % of all adult malignancies. It is male dominant, and has an increasing rate of incidence in many countries (Hollingsworth et al., 2006, Rini et al., 2009). Obesity (Bjorge et al., 2004, van Dijk et al., 2004), active and passive smoking (Hunt et al., 2005) and end-stage renal failure and renal cystic disease are established risk factors for RCC (Ishikawa et al., 2003, Rakowski et al., 2006).

RCC is not a one entity but rather a collection of different types of tumors containing specific attributes, such as histological features, clinical phenotypes and possessing distinct genetic characteristics (Zambrano et al., 1999, Renshaw, 2002, Linehan et al.,

2003, Rini et al., 2009). RCC can be classified in several histological subtypes with different prevalence: clear cell 80%, papillary 10%, chromofobe 5% and medullary and collecting tube <1% (Ngo et al., 2014). Clear cell RCC (ccRCC) and papillary RCC (pRCC) are thought to arise from the proximal tubular epithelial cells, whereas collecting duct carcinoma appears from the ducts of Bellini and chromophobe RCC from the distal nephron (Verdorfer et al., 1998, Rini et al., 2009).

The most common subtype of RCC is ccRCC and it is named because of the high content of the cytoplasmic lipids dissolving during the histological preparation and resulting in a clear cytoplasm (Rini et al., 2009). VHL disease is related to the sporadic and hereditary forms of ccRCC (Renshaw, 2002). The second common subtype of RCC is pRCC and it is further divided into type 1 and a more aggressive type 2 pRCCs (Rini et al., 2009). No differences in the 5-year cancer-specific survival has been observed between patients with pRCC and ccRCC (Waldert et al., 2008).

2.2. von Hippel-Lindau (VHL) and hypoxia

VHL is a tumor suppressor gene located in chromosome 3 (3p25-26). *VHL* mutations are responsible for the most common hereditary syndrome in RCC (Rini et al., 2009). Usually the loss of the second allele, mutation or inactivation of the promoter region of *VHL* gene leads to VHL syndrome (Pavlovich et al., 2003). Patients diagnosed with VHL disease usually develop pre-neoplastic renal cysts that can lead to clear-cell type carcinoma (Kaelin, 2002). Besides RCC, *VHL* mutations appear also in highly vascularized haemangioblastoma of the central nervous system and pheochromocytomas (Rini et al., 2009). VHL regulates HIF-1α and HIF-2α, and in normal physiologic conditions and normal oxygen level, VHL protein (pVHL) binds to prolyl-hydroxylated HIF-αs, resulting its ubiquitination and degradation by the proteasome (Bader and Hsu, 2012). In hypoxic conditions HIF-α translocates to the nucleus, dimerizes with HIF-1β, and acts as a transcription factor inducing gene expression of several genes involved in angiogenesis (*VEGF* and *PDGF*), erythropoiesis, glucose uptake and metabolism and transforming growth factor-α (TGFα) (Kaelin, 2002, Kaelin, 2007). When *VHL* gene is mutated, it does not bind hydroxylated HIF-α, which results in nuclear localization of HIF-α, formation of the αβ-dimer and activation of HIF-target genes (Bader and Hsu, 2012).

2.3. Metastatic renal cell carcinoma (mRCC)

In 25-30% of RCC patients, the tumor has metastasized by the time they are diagnosed (Motzer et al., 1996), and 20-40% patients undergoing nephrectomy of the primary tumor will develop metastasis (reviewed in Lam et al., 2005). Usually RCC metastasis are found in the lung parenchyma (50-60%), bone (30-40%), liver (30-40%) and brain (5%) (Motzer et al., 1996). mRCC is highly resistant to chemotherapy and in the majority of the cases to cytokine-based immunotherapy, bur responds to immune check-point inhibitors (Rini et al., 2009, Coppin et al., 2011). Even though seven targeted therapies for mRCC have been approved since 2005, including inhibitors of

VEGF and the mammalian target of rapamycin (mTOR), the prognosis of mRCC is poor and complete durable responses are rare (Escudier et al., 2014b).

2.4. Microvessel density

MVD is the most frequently used parameter to quantify intratumoral angiogenesis (Cheng et al., 2014). Many studies show that tumors with a high MVD are more aggressive and predispose to tumor metastasis (reviewed in Baluk et al., 2005). It has been shown that CD31 expression correlates significantly with poor prognosis and metastasis in ccRCC patients (Joo et al., 2004). Instead, in patients treated with antiangiogenic therapy, Dornbusch et al. (2013) showed that high CD31 expression compared to low CD31 in primary tumors of mRCC patients treated with sunitinib showed better response to treatment. Sharpe et al. (2013) demonstrated that mRCC patients with higher levels of CD31 and Ki-67 were associated with delayed progression. They also showed that tumor vessel density (CD31) was significantly decreased after TKI (sunitinib and pazopanib) treatment in patients treated before nephrectomy, and during the therapy increased CD31 correlated with reduced OS. del Puerto-Nevado et al. (2014) demonstrated that active angiogenesis in RCC patients predicted the benefit to first-line sunitinib. They showed that phosphorylated VEGFR2 in the tumor stroma was associated with significantly prolonged PFS and OS when treated with sunitinib, but MVD did not show statistical difference in terms of PFS and OS.

2.5. Therapeutic options

In localized RCC, the treatment involves partial or radical nephrectomy, and the choice of treatment needs to be individualised (Van Poppel et al., 2011). The treatments of mRCC consist of targeted therapies and surgery, most often the combination of both treatments, but usually patients with recurrent mRCC or whom surgery is not advisable, the systemic treatment is recommended (Rini et al., 2009).

2.5.1. Immunotherapy

The standard care for mRCC was immunotherapy for many years, specifically interleukin-2 (IL-2) and interferon-α, even though the effectiveness of these treatments is disputable (Rini et al., 2009). Also, the adverse effects of IL-2, including capillary leak syndrome, tachycardia, hypertension, dyspnea and pulmonary congestion, gastrointestinal and renal toxicities, limited the use of this therapy (Poust et al., 2013). IL-2 was approved by Food and Drug Administration (FDA) in the US because of its effect in high dose treated mRCC patients: durable complete remission occurred in 7-8% of patients (Fyfe et al., 1995, Fisher et al., 2000, reviewed in Rini et al., 2009). Combination of low-dose IL-2 and interferon treatment provided prolonged response rate and PFS compared to either drug alone but it did not affect to OS (Negrier et al., 1998). It has been showed that high level of carbonic anhydrase-9 (CAIX), a VHL mediated protein and a target of hypoxic VHL signalling pathway, is associated with poor prognosis in RCC (Bui et al., 2003). In addition, it has been

demonstrated that CAIX might be a predictive biomarker to high dose IL-2 treatment and patients with high level of CAIX might benefit the treatment (Atkins et al., 2005). Dudek et al (2010) showed that mRCC patients without tumor CAIX expression detected by IHC did not response to IL-2 therapy. Instead, recently published results show that durable and objective response to IL-2 therapy did not associate with high tumoral CAIX expression (McDermott et al., 2015).

Interferon-α has provided survival combined with medroxyprogesterone acetate and vinblastine when compared to treatments alone (Pyrhonen et al., 1999, Medical research council renal cancer collaborators, 1999). Instead, no benefit on PFS or OS was registered in interferon-α or IL-2 treated mRCC patients compared to medroxyprogesterone, and toxicity grade was significantly higher in cytokine-treated patients compared to medroxyprogesterone-treated ones (Negrier et al., 2007). More investigation is needed to select the patients who benefit from treatment, and in addition, more identification of clinical and biological predictors to response cytokines are needed. Interestingly, in randomized, double blind phase III trial, interferon-α combined with targeted therapies in mRCC patients showed a significant improvement in PFS when compared to interferon-α treatment alone (Escudier et al., 2007b).

2.5.2. Tyrosine kinase inhibitors (TKIs)

An increased understanding of tumor biology and molecular signalling pathways has established the development of targeted therapies in RCC. For example, the differences in protein levels in RCC caused by *VHL* gene mutations resulted in the understanding of the function of VEGF and mTOR as relevant targets in RCC (reviewed in Rini et al., 2009). Drugs that target these pathways have been developed against the VEGF ligand or VEGFRs, and seven of these targeted drugs have been approved by European Medicines Agency (EMA) and the FDA for the use of mRCC (Escudier et al., 2014b).

Sunitinib is a multi-targeted TKI, which FDA authorized for treatment of mRCC on January 26, 2006. It is now widely used as a first-line therapy for mRCC. Sunitinib is an oral drug that inhibits VEGFRs, PDGFRa/b, KIT, Flt-3 and CSF-1 (Powles et al., 2011). Previous studies have shown ORRs of 25-47% to sunitinib in mRCC, however complete durable responses are rare (Motzer et al., 2006). In addition, approximately 11 months after the initiation of sunitinib treatment disease progression usually occurs (Motzer et al., 2009b). In phase III clinical trials, sunitinib treatment compared to interferon-α increased median PFS of RCC patients, 11 months vs. 5 months, respectively (Motzer et al., 2007). In the same study, median OS was greater in sunitinib treated (26.4 months) than interferon-α group (21.8 months) (**Table 1.**) (Motzer et al., 2009b). Also, the quality of life was better in sunitinib treated patients (Motzer et al., 2007), even though adverse effects such as hypertension (12%), fatigue (11%), diarrhea (9%), and hand-foot syndrome (9%) were reported in the sunitinib arm (Motzer et al., 2009b). In addition, not all patients benefited from the sunitinib treatment (Motzer et al., 2009b, Busch et al., 2011). To improve efficacy,

investigational Ang1/Ang2 blocking peptibody trebananib, which inhibits the interaction of angiopoietin and Tie2, has been combined with sunitinib in clinical phase II RCC trials (Atkins et al., 2015). In this study, the dose effect of trebananib (low dose: group A, high dose: group B) to survival of mRCC patients was investigated. Trebananib treatment was combined with sunitinib. The ORR was 58% in group A, and 63% in group B. PFS was prolonged in group B, when compared to group A (16.3 months vs. 13.9 months, respectively). Although the efficacy results show a benefit for the higher dose of trebananib, the combination therapy with both doses (high and low doses of trebananib) increased the drug toxicity: more than half of patients in both group (high and low doses of trebananib) had sunitinib interruption, and most common adverse events were diarrhea, mucosal inflammation and hypertension (Atkins et al., 2015).

Pazopanib is an oral angiogenesis inhibitor that blocks VEGFRs, PDGFRs and c-Kit (Sternberg et al., 2010). In a randomized, double blind, placebo controlled clinical phase III trial patzopanib showed similar efficacy than sunitinib (Sternberg et al., 2010). In this study RCC patients, including treatment-naïve or previously treated with pazopanib, were randomized to receive placebo or pazopanib. Overall population PFS was 9.2 months vs. 4.2 months in pazopanib treated vs. placebo, respectively. In addition, in cytokine pre-treated group PFS was longer in pazopanib treated than in placebo group (7.4 months vs. 4.2 months), as well as without cytokine treatment (11.1 months vs. 2.8 months). Adverse events, such as diarrhea, hypertension, nausea, anorexia and vomiting were reported (Sternberg et al., 2010). OS was not statistically significant in patzopanib treated compared to placebo group (22.9 months vs. 20.5 months, respectively) (**Table 1.**) (Sternberg et al., 2013). The safety and efficacy of pazopanib and sunitinib as first line therapy were compared in a randomized phase III trial (Motzer et al., 2013b). The results demonstrated similar results between patients receiving pazobanib and sunitinib: The median PFS was 8.4 months in pazopanib treated, and 9.5 months with sunitinib group. Also, there was no difference in OS (pazopanib 28.4 months vs. sunitinib 29.3 months). Instead, pazopanib was better endured: the quality of life and safety of the treatment favoured pazopanib (Motzer et al., 2013b). Supporting these results, recently published double-blind cross-over study of patient preference or quality of life for sunitinib or pazopanib demonstrated that pazopanib was the more preferred treatment (61% physicians, 70% patients) (Escudier et al., 2014a).

Bevacizumab is a monoclonal Ab to inhibit VEGF (Escudier et al., 2007b), and it has shown clinical benefit in phase II trials: PFS was significantly longer in the patient group treated with bevacizumab than in placebo group (4.8 months vs. 2.5 months, respectively) (Yang et al., 2003). Afterwards, in a randomized double-blind phase III trial bevacizumab treatment was combined with interferon-α, and compared with placebo and interferon-α (Escudier et al., 2007b). The median PFS was significantly longer in bevacizumab plus interferon-α treated (10.2. months) than placebo plus interferon-α treated (5.4 months) patient group. Instead, the median OS was not significantly longer between bevacizumab (23.3 months) or placebo (21.3 months) groups (**Table 1.**) (Escudier et al., 2010). In another phase III trial OS, ORR and safety

of bevacizumab plus interferon-α treatment was compared to interferon-α alone (Rini et al., 2010). Also in this study, median OS was not significantly longer in bevacizumab plus interferon-α treated (18.3 months) than in control group (17.4 months). Interestingly, patients receiving bevacizumab plus interferon-α treatment and with hypertension, had significantly improved PFS and OS compared with patients without hypertension, suggesting that hypertension might be a potential biomarker in bevacizumab targeted therapies (Rini et al., 2010).

Sorafenib is an oral multikinase inhibitor for VEGFR, PDGFRβ, FMS-like tyrosine kinase 3 (Flt-3), c-Kit protein and RET tyrosine kinases (reviewed in Escudier et al., 2007a). In phase III trials, sorafenib was effective compared to placebo control group. PFS was 5.5 months in sorafenib treated and 2.8 months in placebo group, partial response (PR) reports as the best response, but no difference in OS (19.3 with sorafenib vs. 15.9 months with placebo) (**Table 1.**) (Escudier et al., 2007a). In another phase II trial, where the effect of sorafenib was compared with interferon-α or treatment-naïve RCC patients, PFS was similar between sorafenib and interferon-α treated groups. Instead, in sorafenib treated group, tumor size reduction was detected and the quality of life was better (Escudier et al., 2009). Because of inefficiency of sorafenib, the European Society of Clinical Oncology (ESMO) has recommended to use a combination of sorafenib and interferon-α as a front-line treatment (Escudier et al., 2014b).

Axitinib is a second-generation VEGFR1, -2, and -3 inhibitor (Rini et al., 2011). In a phase III randomized trial the effect and safety of axitinib and sorafenib as a second-line therapy were compared in mRCC patients treated first-line with sunitinib, bevacizumab plus interferon-α, temsirolimus, or cytokines (Rini et al., 2011). The median PFS was significantly extended with axitinib (6.7 months) compared to sorafenib (4.7 months). The most common adverse events in the axitinib treated patients were diarrhoea, hypertension, and fatigue, and in the sorafenib treated group diarrhoea, palmar-plantar erythrodysaesthesia, and alopecia (Rini et al., 2011). No difference in OS was detected between axitinib (20.1 months) and sorafenib (19.2 months) treated groups (**Table 1.**) (Motzer et al., 2013a). In addition, there was no difference in PFS between axitinib and sorafenib groups in first-line treated RCC patients (Hutson et al., 2013).

2.5.3. Mammalian target of rapamycin (mTOR) inhibitors

The activity of the mTOR is tightly regulated in normal cells, but its activity is abnormal in tumors (Vogt, 2001). mTOR is a one of the key components and regulators of intracellular pathways such as Akt (cellular survival), PKCα (cytoskeletal dynamics), serine/threonine-protein kinase (SGK1, ion transport and growth), and mTOR participates in tumor cell proliferation, survival, growth and also angiogenesis (Kapoor and Figlin, 2009). mTOR activity is increased by PI3K/Akt, EGF and Ras/MAPKs during tumorigenesis (Vogt, 2001, reviewed in Kapoor and Figlin, 2009). Activated mTOR induces the phosphorylation of components of the eukaryotic initiation factor 4E-binding protein 1 (4EBP-1) and the translation-

regulating factors ribosomal S6 kinase 1 (S6K1), which participate in cell proliferation and growth (Gingras et al., 1998, Kapoor and Figlin, 2009). In addition, in the absence of VHL, activated mTOR further increases the HIF-1α level via its elevated translation, resulting in increased VEGF transcription (Kapoor and Figlin, 2009). Hence, mTOR is a potential target for targeted anti-angiogenic therapies, and at the present, two rapamycin-related mTOR inhibitors temsirolimus and everolimus are now showing significant activities against RCC, and are approved for the RCC treatment.

Temsirolimus is a mTOR inhibitor, and in phase III trial its efficacy compared to interferon-α, or combination of both treatments (Hudes et al., 2007). Temsirolimus treatment alone improved OS and PFS: the median survival was 10.9 months in temsirolimus treated group, 7.3 months in the interferon-α treated and 8.4 months in the combination group (**Table 1.**), and PFS was in the temsirolimus treated 3.8 months, interferon-α treated 1.9 months, and combination treatment 3.7 months. In another study, the effect of temsirolimus to different tumor histology types was compared with interferon-α treatment (Dutcher et al., 2009). Temsirolimus had a similar response rate compared to interferon-α, but interestingly, in temsirolimus treated patients tumor reduction was reported to be 59% with clear cell, and 68% with other tumor type vs. 35% with clear cell and 14% in other histologies in interferon-α treated patients (Dutcher et al., 2009). Temsirolimus is recommended by ESMO and National Comprehensive Cancer Network (NCCN) as first-line treatment for RCC patients with poor prognosis (Motzer et al., 2009a, Escudier et al., 2014b).

Everolimus is an orally administered derivative of rapamycin that forms a complex with an intracellular protein FKBP-12 resulting in mTOR inhibition. Everolimus is a sequential therapy with promising efficacy for RCC patients with progressed disease after the failure of TKI treatments (Motzer et al., 2008). In a phase III randomized double-blind placebo controlled trial of everolimus in patients treated with first-line sunitinib or sorafenib whom disease had progressed, the PFS was prolonged in everolimus treated group (Motzer et al., 2008). Instead, everolimus did not prolong the median OS (14.8 months with everolimus vs. 14.4 months with placebo) (**Table 1.**) (Motzer et al., 2010).

Table 1. List of tyrosine kinase- and mTOR inhibitor treatments and trials in RCC.

Treatment	Control group	Trial	End point	Results (months)
TKI Inhibitors				
Sunitinib	Interferon-α	Phase III	OS	26.4 vs. 21.8
Pazopanib	Placebo	Phase III	OS	22.9 vs. 20.5
Bevacizumab + Interferon-α	Interferon-α + Placebo Interferon-α	Phase III Phase III	OS OS	23.3 vs 21.3 18.3 vs 17.4
Sorafenib	Placebo	Phase III	OS	19.3 vs. 15.9
Axitinib	Sorafenib	Phase III	OS	20.1 vs. 19.2
mTOR Inhibitors				
Temsirolimus	Interferon-α	Phase III	OS	10.9 vs. 7.3
Everolimus	Placebo	Phase III	OS	14.8 vs. 14.4

2.6. Prognostic and predictive biomarkers of renal cell carcinoma

Advances in diagnosis and discovery of a disease may be the reason for the increased incidence of RCC. Increased use of cross-sectional imaging facilitates the detection of small incidental carcinomas. Better understanding of genomics, heredity, proteomics and metabolomics of RCC has shed light for the development of new-targeted therapies, as well as diagnostic, prognostic, and predictive biomarkers. Many candidate biomarkers show promising results in current studies, but so far none has entered clinical use, and little is known who will benefit from RCC targeted therapies (Ngo et al., 2014).

VHL is a potential biomarker because of its downstream proteins are involved in angiogenesis, but the predictive value of *VHL* mutation and outcome is contradictory. Poor OS and PFS have been reported in patients with loss-of-function *VHL* mutations (Schraml et al., 2002). Instead, there was no association with VHL alteration and MVD, tumour cell proliferation, grade and stage (Schraml et al., 2002). In another study, patients with sporadic ccRCC and loss-of-function *VHL* mutation were found to have a significantly decreased OS (P= 0.046) and PFS (P= 0.016), but instead, there was no association between somatic *VHL* alteration and PFS and OS (Kim et al., 2005a). Smits et al. (2008) also found no prognostic value in alterations of *VHL* mutation in RCC. Instead, Brauch et al. (2000) demonstrated that *VHL* alterations associate significantly with poor staging (pT3) indicating the possibility of VHL to be a prognostic biomarker. Choueiri et al. (2008) demonstrated that loss-of-function *VHL* mutations could be a prognostic or predictive biomarker, because patients with *VHL* mutations responded better to VEGF therapy, even though the association was not significant.

VEGF pathway is a potential biomarker, and high VEGF expression in plasma and tumor tissue has been reported to correlate with tumor size, stage and grade (Jacobsen et al., 2004, Rioux-Leclercq et al., 2007). Also, VEGF expression in tumor was correlated with tumor necrosis and progression (Rioux-Leclercq et al., 2007). Plasma levels of VEGF, soluble VEGFR2 (sVEGFR2), PlGF, and a soluble VEGFR3 (sVEGFR3) were analysed from the patients treated with sunitinib, and levels were varied according the treatment: 2 weeks after off treatment, the levels of the above mentioned proteins tended to return to near-baseline (Deprimo et al., 2007). In another study, the effect of sunitinib to the bevacizumab-refractory mRCC patients was investigated. In this study, VEGF-C and sVEGFR3 levels were decreased with sunitinib treatment and associated with longer PFS and ORR (Rini et al., 2008). Recently, in a phase III trial, Harmon et al. (2014) investigated potential circulating biomarkers to predict the efficacy of first-line sunitinib versus interferon-α in mRCC. They found, that baseline VEGF associated with PFS, and sVEGFR3 associated with both, PFS and OS in sunitinib treated patients. In interferon treated patients, IL-8 was associated with PFS. These results indicate that baseline sVEGFR3 may predict sunitinib efficacy, and baseline VEGF and IL-8 may have a prognostic value. In another study, VEGFR2 expression in tumor tissue was significantly associated with PFS and response to sunitinib treatment (Terakawa et al., 2013),

whereas Gruenwald et al. (2010) showed that sVEGFR2 failed to correlate with clinical response in sunitinib treated patients.

CAIX shows as a promising biomarker as discussed earlier, instead, Leibovich et al. (2007) demonstrated that CAIX is not an independent prognostic marker in RCC patients. In addition, in another study serum CAIX level was higher in ccRCC patients but did not correlate to survival (Papworth et al., 2010).

Ki-67 is a cell proliferation marker in oncology and especially in RCC, it has raised interest as a prognostic biomarker (Ngo et al., 2014). Few studies have demonstrated that Ki-67 predicts poor prognosis in RCC patients (Tollefson et al., 2007, Rautiola et al., 2016, Lampinen et al., 2016).

Cytokine and angiogenic factors produced by tumors have been speculated to participate in the disease progression after antiangiogenic therapies (Ngo et al., 2014). *In vivo* study using mouse xenografts demonstrated that increased IL-8 expression was associated with sunitinib resistance, and that was inhibited by blocking IL-8 (Huang et al., 2010). In humans in a clinical phase II trial of pazopanib, PFS and tumor shrinkage was associated with IL-6, IL-8, osteopontin, VEGF, E-selectin and hepatocyte growth factor (HGF) levels (Tran et al., 2012). High concentrations of IL-8, osteopontin, tissue inhibitor of metalloproteinases (TIMP) -1 and HGF were associated with shorter PFS (Tran et al., 2012). In a phase II trial of sorafenib, and sorafenib plus interferon-α treatment, cytokine and angiogenic factors were investigated in mRCC patients, demonstrating correlation of prolonged PFS with osteopontin, VEGF, CAIX, collagen IV, VEGFR-2, and tumor necrosis factor-related apoptosis-inducing ligand levels (Zurita et al., 2012).

Genetic variations, such as single nucleotide polymorphisms (SNPs), have been identified as biomarkers for the treatment. Patients with SNPs in *IL-8*, *HIF-1A* and *VEGF* compared to patients without SNPs had lower response rates and reduced PFS when treated with pazopanib (Xu et al., 2011). Garcia-Donas et al. (2011) demonstrated that two SNPs in *VEGFR3* were associated with reduced PFS in RCC patients treated with sunitinib. Interestingly, it has been shown that SNPs in three genes involved in sunitib pharmacokinetics (*ABCB1, CYP3A5, NR1I3*), which for example participated in the metabolism of TKI, improved PFS (van der Veldt et al., 2011). In addition, methylation of CpG islands in five different genes (*PITX1, FOXE3, TWF2, EHBP1L1, RIN1*) could help to predict OS in patients categorised to low and high risk groups (Wei et al., 2015). Also, micro RNAs (miRNAs) have been involved in several tumorigenic pathways and recently published data suggest that five circulating miRNAs can be used clinically in early detection of RCC (Wang et al., 2015). In addition, Gu et al. (2015) demonstrated that miRNAs, especially miR-21 and miR-126 could be prognostic markers in RCC and also, useful therapeutic targets.

AIMS OF THE STUDY

The aim of the study was to investigate the Ang-Tie system at the molecular level to better understand the mechanisms that lead to vessel stabilization, and those that lead to leaky vessels involved in tumor angiogenesis and metastasis. Identifying Ang/Tie signalling mechanisms should increase the possibilities of targeting the Ang-Tie system in anti-angiogenic therapies for the treatment of vascular pathologies and cancer. In addition, the determination of disease-specific expression of the Ang-Tie pathway components should facilitate their therapeutic targeting and use as biomarkers in human disease.

Specific aims are:

1) *Discover the function of Tie1 and integrins in angiopoietin signalling and Tie1-mediated regulation of Tie2 in ECs using cell biological and microscopic techniques, including fluorescence resonance energy transfer (FRET-FLIM-TCSCP) (publication III).*

2) *Investigate Ang2 as a potential prognostic and predictive biomarker in mRCC (publications I and II)*

3) Investigate the mechanism of action of a therapeutic Ang2 blocking Ab in tumor metastasis (publication IV).

PATIENTS, MATERIALS AND METHODS

Detailed description of the materials and methods can be found in the original publications (I-IV).

The HUCH Ethics Committee (I), and the Ethics Committee at Tampere University Hospital (II) and the National Authority for Medicolegal Affairs (II) approved the use of tumor samples and the research protocol.

All experiments that required animal usage were approved by the National Animal Experiment Board in Finland at the Provincial State Office of Southern Finland, and were performed in accordance with the Finnish legislation regarding use of laboratory animals.

1. Patients and treatments

1.1. Patients in publication I

This study contained clinical samples from 136 surgically removed primary tumors from mRCC patients who received first-line sunitinib treatment between 2006 and 2012 in the Department of Oncology, Helsinki University Central Hospital (HUCH). No prior targeted therapies were approved. Follow-up data of response to sunitinib treatment was available from 126 patients. All clinical data and follow-up record were collected from the hospital case records.

1.2. Patients in publication II

This study contained clinical RCC tumor samples from 244 RCC patients, who were treated by the same clinical practice, and underwent nephrectomy between 1985 and 1995. Tumor samples were obtained from the archives of Tampere University Pathology, and were re-evaluated using the Furman grading system and re-classified using Heidelberg classification system.

2. Materials

2.1. Cell lines

Table 2.

Cell line	Description	Medium	Source	Used in
HUVEC	Human umbilical vein endothelial cells	ECBM-MV + 5% FCS	PromoCell	III, IV
BEC	Human dermal blood endothelial cells	ECBM-MV + 5% FCS	PromoCell	IV
LEC	Human dermal microvascular lymphatic endothelial cells	ECBM-MV + 5% FCS	PromoCell	IV
NCI-H460-LNM35	Human non-small cell lung carcinoma	RPMI-1640 + 10% FCS	(Kozaki et al., 2000)	IV

2.2. Primary antibodies

Table 3.

Target	Description	Application	Source	Used in
Ang2	Goat polyclonal	IHC, WB	R&D Systems	I-IV
Akt	Rabbit polyclonal	WB	Cell Signalling	III
α_5-integrin	Goat polyclonal	WB	R&D Systems	III
β-actin	Rabbit polyclonal	WB	Cell Signalling	III, IV
β_1-integrin	Mouse monoclonal	WB	Merk Millipore	III
β_1-integrin	Mouse monoclonal	IF	Abcam	III
β_3-integrin	Rabbit monoclonal	WB	Abcam	III
BCL-2	Mouse monoclonal	IHC	DAKO	II
CD11b	Rat monoclonal	IF	eBioscience	III
CD31	Mouse monoclonal	IHC	DAKO	I
CD31	Mouse monoclonal	IHC	Novocastra Laboratories	II
mCD31	Hamster monoclonal	IF	Chemicon	III
mCD31	Rat polyclonal	IF, IHC	BD Biosciences	III, IV
Cytoceratin7	Rabbit monoclonal	IHC	Millipore	IV
EEA1	Rabbit monoclonal	IF	Cell Signalling	III
eNOS (pS1177)	Rabbit polyclonal	IF	Cell Signalling	IV
mEphB4	Goat polyclonal	IF	R&D Systems	III
Flag-tag	Rabbit polyclonal	WB	Sigma-Aldrich	III, IV
Foxo1	Rabbit monoclonal	IF	Cell Signalling	III
Human anti-Ang2	Monoclonal, humanized	IF	(Leow et al., 2012)	IV
Human anti-Ang2	Monoclonal, humanized	IF	(Daly et al., 2013)	III
Human anti-Tie2	humanized	IF	(Adler et al., 2014)	III
HSC70	Mouse monoclonal	WB	Santa Cruz Biotechnology	III
Ki-67	Rabbit monoclonal	IF	Abcam	III
Ki-67	Mouse monoclonal	IHC	Immunotech SAS	II
Ki-67	Mouse monoclonal	IHC	DAKO	I
NG-2	Rabbit polyclonal	IHC	Millipore	IV
Phospo-Akt (Ser473)	Rabbit polyclonal	WB	Cell Signalling	III
Phospho Tie2	Rabbit polyclonal	IF	R&D Systems	III, IV
Phospho Tyr	Mouse monoclonal	WB	Merck Millipore	III
Phospho Tyr	Mouse monoclonal	WB	Santa Cruz Biotechnology	III
mP-selectin	Goat polyclonal	IF	R&D Systems	III
Tie1	Goat polyclonal	IF, WB	R&D Systems	III, IV
Tie1	Rabbit polyclonal	WB	Santa Cruz Biotechnology	III
Tie2	Goat polyclonal	IF, IP, WB	R&D Systems	III, IV
m/rTie2	Goat polyclonal	IF, IP, WB	R&D Systems	III
V5-Cy3	Mouse monoclonal	IF	Sigma Aldrich	III
VEGF R2/KDR/Flk-1	Polyclonal Goat	IP, WB	R&D Systems	III
vWF	Rabbit polyclonal	IF	DAKO	III
VE-cadherin	Mouse monoclonal	IF	BD Biosciences	III, IV
VE-cadherin	Rat (clone 11D4.1)	IF	BD Biosciences	IV
ZO-1	Rabbit polyclonal	IF	Invitrogen	IV

2.3. Lentiviruses

Table 4.

Target	Description	Sequence in 5'-3' direction	Used in
β_1-Integrin	shRNA, cloned in pLKO.1 vector	GCCTTGCATTACTGCTGATAT	III
β_3-Integrin	shRNA, cloned in pLKO.1 vector	CCTTAGCCTTTGTCCCAGAAT	III
α_5-Integrin	shRNA, cloned in pLKO.1 vector	CCATGATGAGTTTGGCCGATT	III
Tie1	shRNA, cloned in pLKO.1 vector	GACTGGAGCAACACAGTAGAA	III

2.4. Retroviruses

Table 5.

Retrovirus	Description	Source	Used in
FL-Tie2-GFP	Encodes for C-terminal Tie2-GFP fusion protein	(Saharinen et al., 2008)	III, IV
Tie2-ΔJM-GFP	Encodes Tie2 ectodomain	(Hakanpaa et al., 2015)	III
Tie1-ΔIC-V5	Encodes Tie1 ectodomain	(Korhonen et al., 2016)	III
VE-cadherin-V5	Encodes VE-Cadherin-V5 tag fusion protein	(Korhonen et al., 2016)	III
Tie1-mCherry	Encodes for C-terminal Tie1-mCherry fusion protein	(Korhonen et al., 2016)	III

2.5. Recombinant proteins

Table 6.

Recombinant protein	Description	Source	Used in
rhAng1	Recombinant human Angiopoietin-1	R&D Systems	III
rhAng2	Recombinant human Angiopoietin-2	R&D Systems	III
Comp-Ang1	The N-terminal portion of Ang1 replaced with the short coiled-coil domain of COMP	(Cho et al., 2004), a kind gift from Dr. Koh, KAIST	III
VEGF	Human VEGF	A kind gift from Dr. Michael Jeltsch, Helsinki	III

2.6. Mouse lines and viruses

Detailed description of mouse lines and viruses used in mouse experiments are found in the original publications (III, IV).

Table 7.

Mouse line	Description	Source	Used in
VE-cadherin-tetracyclin transactivator (tTA)	Overexpresses tTA under the *Cdh5* promoter in ECs	(Sun et al., 2005)	III, IV
TetO-Ang2	Inducible Ang2 expression driven by tTa	(Holopainen et al., 2012)	III, IV
Tie1 $^{fl/fl}$	loxP-flanked 1st exon of the *Tie1* gene	(D'Amico et al., 2014)	III
Tie1$^{LacZ/+}$	*Tie* promoter-driven β-galactosidase (Lacz)	(Puri et al., 1995)	III
Pdgfb-iCreER^{T2}	Tamoxifen-inducible form of Cre recombinase under *Pdgfb* promoter	(Claxton et al., 2008)	III
Cdh5(PAC)-CreER^{T2}	Tamoxifen-inducible Cre deletor under the VE-cadherin (*Cdh5(PAC)*) promoter	(Wang et al., 2010)	III
Tie1-tTA	Overexpresses tTA under *Tie1* promoter in EC	(Sarao and Dumont, 1998)	III

2.7. Cell culture (III, IV)

EC lines (**Table 2.**) were maintained in the appropriate Endothelial Cell Basal Medium (ECBM) MV (PromoCell) containing the following supplements: 5% Fetal Calf Serum (FCS), hEGF (10 ng/ml), Hydrocortisone (1 µg/ml), and Endothelial Growth Supplement 0.4% (PromoCell). LECs were grown in ECBM-MV media supplemented with VEGF (100 ng/ml). Cell culture plates were coated with gelatin (0.1%) for HUVECs and LECs, and with fibronectin (1 µg/ml) for blood vascular endothelial cells (BECs).

3. Methods

3.1. Cell stimulations (III, IV)

Confluent cells were starved for 3-5 hours in ECBM-MV media containing 1% FCS (PromoCell). After starvation, cells were treated with Ang1 (100-500 ng/ml), Ang2 (100-500 ng/ml) or Comp-Ang1 (200-500) ng/ml.

3.2. Viral vector delivery (III, IV)

Approximately 50% confluent ECs were transduced with retroviral vectors expressing genes of interest (**Table 5.**) in the presence of 0.1% polybrene (Sigma-Aldrich). 5 hours after adding of the viral vectors, culture media was added, and fresh media was changed after overnight incubation. Lentiviral vectors coding for shRNA were used to

silence genes of interest in 50-90% confluent EC cultures in the presence of 0.1% polybrene (Sigma-Aldrich) (**Table 4.**). Cells were supplied with fresh media next day after transduction.

3.3. *In vivo* treatments (III, IV)

The *Tie1*$^{fl/fl}$, *Tie1*$^{LacZ/+}$, *Pdgfb-iCreER*^{T2}, *Cdh5(PAC)-CreER*^{T2}, *VE-cadherin-tTA (Cdh5-tTA), Tie1-tTA* and *tetO-Ang2* were used in original publication III (**Table 7.**). Inducible inactivation of Tie1 (*Tie1*$^{fl/fl}$, *Tie1*$^{LacZ/+}$) was performed with endothelial-specific deletors *Pdgfb-iCreER*^{T2} and *Cdh5(PAC)-CreER*^{T2} by daily administration of tamoxifen (2mg/mouse/d) for 5 days, and Cre-positive *Tie1*$^{fl/fl}$, *Tie1*$^{LacZ/+}$ were compared with Cre-negative *Tie1*$^{fl/fl}$, *Tie1*$^{LacZ/+}$ mice.

Double-transgenic Ang2 overexpressing mice were obtained via breeding the driver *Cdh5-tTA* or *Tie1-tTA* with the responder *tetO-Ang2* transgenic mouse line (III, IV) (**Table 7.**). Administration of 2 mg/ml of tetracycline in 5% sucrose in the drinking water of pregnant females was used to suppress Ang2 expression. Expression of Ang2 was induced for 2 to 3 months after birth, and serum Ang2 level was increased 25 fold compared to WT mice. WT or single transgenic mice were used as controls (III, IV). *Tie1* deletion in *Ang2*^{EC} mice was obtain by tamoxifen administration 3 times per week for 2-3 weeks to *Pdgfb-iCreER*^{T2} *Tie1*$^{fl/fl}$ *Cdh5-tTA tetO-Ang2* mice (III).

Ad-control (empty control or LacZ), Ad-Comp-Ang1 or Ad-Ang2 (mAng2) adenovirus vectors (1 x 10^9 PFUs/mouse) in 100 µl PBS were injected into tail vein of control or *Tie1*-deficient mice, and after 2 days or 2 weeks, tracheas, lungs and serum were collected for analysis (III).

LPS (15 mg/kg, *E.coli* LPS055:B5, Sigma-Aldrich) i.p. injection was used to induce the endotoxemia in mice, and 0.5, 1, 3, 6, 12 or 16 hours after LPS treatment mice were sacrificed (III).

3.4. Cell sorting and flow cytometry (III)

For FLIM, HUVECs transduced with retroviral vectors coding for the GFP tagged Tie2 ectodomain, were sorted using fluorescence-activated cell sorting (FACS) Aria II Flow Cytometer according to hTie2 expression levels. In brief, HUVECs were detached using Accutase (Biosera) for 2 minutes, washed with Dulbecco's phosphate-buffered saline (DBPS, Lonza), sentrifuged 1300 rpm for 3 min, and suspended in PBS (filtered to the FACS tube) for FACS sorting. HUVECs with low expression level of Tie2 (lowest 30% of the transduced HUVEC population) were plated and transduced with a retroviral vector coding for the V5 tagged membrane-bound Tie1 ectodomain. Transduced cells were FACS sorted according to Tie1, by staining the cells using the anti-hTie1 Ab (R&D Systems) diluted in 1% Bovine Serum Albumin (BSA) (Biotop Oy) in PBS for 20 min on ice. The cells were washed with PBS, and incubated with secondary Alexa Fluor 647 Donkey Anti-Goat IgG (Life Technologies) diluted to 1% BSA-PBS on ice for 15 min, washed with PBS and

subjected to FACS sorting. HUVECs, which expressed medium levels of Tie1 (mid 30% of the cell population) were used for FLIM experiments.

3.5. Immunofluorescence staining (III, IV)

Cells plated on cover slips were fixed with 4% PFA (paraformaldehyde), permeabilized (0.1% Triton-X in PBS), and blocked with 1% BSA-PBS, and incubated with primary Abs (**Table 3.**) diluted in blocking buffer. Subsequently, the cells were stained with fluorescently labelled secondary Abs (Alexa Fluor, Life Technologies) and for nuclei using Hoechst and mounted using Mowiol-Dabco (Sighma-Aldrich) or Vectashield (Vector Laboratories) with DAPI (IV). For FLIM and FRET (III), cells were stained with anti V5-Cy3 labeled Ab (Sigma-Aldrich) and mounted with Vectashield (Vector Laboratories) without nuclear staining.

3.6. Immunoprecipitation and Western blot (III)

ECs were lysed in a lysis buffer including 5% glycerol, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 100 nM NaF, 10 mM $Na_4P_2O_7 \cdot 10\ H_2O$, 50 mM Hepes, 1 mM Na_3VO_4, PMSF, leupeptin and approtinin. Protein concentration was analysed using the Pierce BSA Protein Assay kit (Thermo Fisher Scientific). Equal amount of protein was immunoprecipitated over night with primary Abs (**Table 3.**), and using protein G-sepharose for one hour (GE Healthsciences Ab). Protein complexes or total lysates were separated with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (7.5%, 4-20% gradient Ready-Gels, Bio-Rad) and transferred to PVDF membrane (TransBlot Turbo Midi-size, Bio-Rad) using Transblot Turbo (Bio-Rad) transfer system with manufacturer's instructions. Membranes were blocked (5 % BSA in PBS + 0.1% Tween) and incubated over night with primary Abs (**Table 3.**) at +4°C, washed, blocked and incubated with biotinylated secondary Abs and with streptavidin horseradish peroxidase (HRP) (GE-Healthcare), or with HRP-conjugated secondary Abs. Signal was detected with SuperSignal West Pico or SuperSignal West Femto kit (Thermo Fisher Scientific)

3.7. Microscopy, confocal microscopy, live cell imaging and image analysis (I-IV)

Bright field images from IHC sections (I) were acquired using a Leica DM LB microscope (Meyer Instruments) with Olympus DP50 camera using 10x (NA 0.25), 20x (NA 0.4) and 40x (NA 0.65) objectives with magnification x100, x200 and x400, respectively.

Bright field images from the IHC tissue microarray (TMA) sections (II) were scanned using the Panoramic FLASH II (3DHISTECH Ltd) with 40x objective. The HistoQuant image analysis software module (3DHISTECH) was used to measure the Ang2 positive area from digital images. Nonspecific or low quality of staining (broken or detached tissue) was excluded.

Immunofluorescent samples were imaged using a confocal laser-scanning microscope (LSM) Zeiss LSM 780 or LSM 880 with Airyscan (III) using the Zeiss ZEN 2 software, or Zeiss LSM 5 Duo (IV) confocal microscope (Carl Zeiss). 3-dimensional projections were digitally reconstructed from confocal z-stacks using the LSM software. ImageJ (National Institutes of Health, USA) software was used for area-based image quantifications.

Time-lapse imaging (III) was performed using Zeiss LSM 880 (Carl Zeiss) equipped with an environmentally controlled incubator (CO_2 5%, +37ºC) (PM S1), with 63x Plan Apochromat Oil-immersion objective (NA 1.40) and time-lapse Z-stack images were taken every 20 seconds. Z-stacks were imaged before and after stimulation. Time-lapse movies were created using Zeiss ZEN 2 software.

3.8. FLIM and FRET (III)

FRET acceptor photo bleaching was performed using Leica TCS SP2 (Leica Microsystems) confocal microscope with 63x HCX PL APO CS oil-immersion objective NA 1.40 or Leica TCS SP8 (Leica Microsystems) confocal microscope with 63x HC PL APO CS2 oil-immersion objective NA 1.30 (β_1-integrin silenced cells). Excitation wavelengths of 488 nm (argon laser) and of 561 nm (DPSS laser) for GFP and Cy3, respectively, were used. Fluorescence emissions at 500–530nm for GFP, and 570–632nm for Cy3 were recorded. Regions of interest (ROI) were chosen after angiopoietin stimulation at EC-EC junctions and on cell membrane. For acceptor photobleaching ROI was imaged at 100% of DPSS laser power as long as the Cy3 fluorescence intensity decreased to 0-15% intensity (monitored by the Leica software). Pre- and post-bleach images were recorded for both donor (GFP) and acceptor (Cy3). FRET efficiency was calculated as FRETEff = (Dpost − Dpre) / Dpost, where Dpre and Dpost is the donor (GFP) intensity before and after acceptor (Cy2) photo bleaching. When Dpost > Dpre, the FRETEff was considered as a positive. GFP intensity was verified outside the bleached area before and after bleaching.

3I Marianas (3I intelligent Imaging Innovations) fluorescence microscope with 63x Alpha Plan-Apochromat Oil immersion objective NA 1.46 and 488nm solid-state laser modulated at 50 MHz was used to perform Frequency Domain FLIM. Semrock LF488-B filter set was used to exite the donor (GFP). Images were processed using Slidebook 5.5 software, and the lifetimes were calculated from selected ROI.

3.9. Time-Correlated Single Photon Counting (TCSPC) (III)

Time-resolved fluorescence measurement was performed using the Zeiss LSM 880 with 63x Plan-Apochromat Oil-immersion objective NA 1.40, equipped with a FRET module. A pulsed PDL 800-D Diode laser driver modulated at a repetition time rate of 40 MHz was the excitation source, and GFP was excited with a wavelength of 485 ± 10 nm (LDH-D Series, PicoQuant). Photon counting was performed until 60 000-100 000 photons were counted. Data analysis was performed using SymPhoTime64 software to measure the fluorescence lifetime of GFP from ROI.

3.10. Immunohistochemistry (I, II)

IHC staining was performed on 5µm, formalin-fixed paraffin-embedded tumor tissues. Tissues were deparaffinized (Tissue-Tek, Tissue Clear Xylene substitute, SAKURA), rehydrated, and stained using the Tyramide Signal Amplification kit (PerkinElmer) following the manufacturer's instructions. Antigen retrieval was executed using 2100-Angtigen Retriever (Aptum Biologics Ltd.) (II) at 120º for 20 minutes, or microwaves (I) at 750 W for 5 min, and subsequently at 450 W for 10 min, in 10 mM citrate buffer (1.8 mM citric acid, 8.2 mM sodium citrate). 10% hydrogen peroxidase were used to block endogenous peroxidase activity, and nonspecific binding was blocked using TNB (0.5%) blocking buffer (Perkin Elmer). Ang2 expression and blood vascular endothelium were detected using human Ang2 (R&D Systems) (**Figure 5.**) and a mouse monoclonal Ab to human CD31 (DAKO (I) or Novocastra Laboratories (II)) (**Table 3.**), and biotinylated anti-goat or anti-mouse secondary Abs (Vector Laboratories) diluted in TNB. Signal was detected using AEC (3-amino-9ethylcarbatzole) chromogen method. Sections were counterstained with Mayer's Hemalum Solution (Merck KGaA).

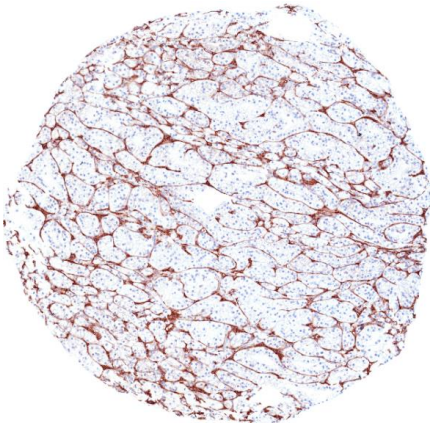

Figure 5. Ang2 immunohistochemical (IHC) staining of tissue microarray (TMA) of renal cell carcinoma (RCC). Ang2 is expressed in tumor endothelium

Ki-67 IHC was performed using a mouse anti-human Ki-67 mAb (KI-67, DAKO (I) or Immunotech SAS (II)) with a Ventana UltraView Dab V3 (Ventana Medical Systems) kit with amplification, and a Ventana Bench Mark XT immunostainer automate (Ventana Medical Systems) (I).

3.11. Statistical analyses

I-II: The IBM SPSS Statistics software for Mac (20.0 (I) and 21.0(II)) (IBM Corporation) was used to perform statistical analyses. Kaplan Meyer method was used to estimate the PFS and OS (I-II). Age- and gender-adjusted univariate and multivariate survival analyses were performed using the Cox proportional hazards model (II), and Pearson χ^2 test was used to analyse the differences between categorical variables (II). Long-rank test was used to analyse and compare survival between the

groups, and Fisher's exact test or Chi-squared test to analysed frequency tables of clinic-pathological parameters (I). All tests were two-tailed (I). *P* values less than 0.05 were considered as statistically significant.

III: SPSS or Prism Statistical (GraphPad Software) analysis was used to perform 1-way ANOVA, followed by Tukey's, Dunnett's or Fisher's least significant difference (LSD) test, or Student's *t* 2-tailed test. Welch's *t* test (2 tailed, unequal variance) followed by Bonferroni's correction was used for pairwise comparisons of FLIM and FRET measurements. *P* values less than 0.05 were considered as statistically significant.

IV: Statistical analysis of metastasis occurrence was performed with Fisher exact test, and the analysis of primary tumor growth curves was performed using one-way analysis of variance. All tests were two-sided. *P* values less than 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

There has been a significant interest to utilize the Ang-Tie system as a target for future anti-angiogenic therapies and several investigational drugs targeting this system are in clinical trials. However, the signalling mechanisms of Angs and the function of the orphan Tie1 receptor are not fully understood, creating uncertainty how to best develop Ang-Tie targeted therapeutics. Identifying the signalling mechanisms of the Ang-Tie pathway should increase the possibilities for its therapeutic targeting for the treatment of vascular pathologies and cancer. Here, we investigated the Ang-Tie system at the molecular level to better understand the mechanisms that lead to vessel stabilization, and those that lead to leaky vessels involved in tumor angiogenesis and metastasis, as well as in inflammation. In addition, determination of the expression of the Ang-Tie pathway components in human disease, including different cancers, will lead to more comprehensive understanding of the role of this vascular system in human disease, potentially guiding future decisions of cancers where the Ang-Tie system might be best targeted.

1. Tie1 participates in angiopoietin signalling *in vivo* and *in vitro* (III)

As described above, Ang1 can act as a Tie2 agonist, which promotes vascular stabilization in cancer, but also in inflammation and sepsis, whereas Ang2 can act as a context-dependent Tie2 agonist or antagonist. In our original publication III we investigated the function of Tie1 in angiopoietin signalling and vascular remodelling. For this we used EC cultures to investigate molecular mechanisms of Tie1-Tie2 signalling complex formation, transgenic mice expressing Ang2 in the vascular ECs, and EC specific *Tie1* deletion in adult mice to study *in vivo* vascular functions of Ang2 and Tie1, and compared the effects with adenoviral delivered Ang1 and Ang2. In addition, we investigated the changes in Tie signalling in inflammation following LPS and TNF-α administration in mice.

2. Tie1 is required for Angiopoietin-mediated vascular remodelling (III)

Previously, it has been shown that adenoviral vector delivery of Ang1 and Ang2 induced tracheal vascular remodelling (Kim et al., 2007). To confirm these results, we injected adenoviral Comp-Ang1 (Ad-CAng1) and native Ang2 (Ad-Ang2) to WT mice and found enlargement of the tracheal vessel after both stimulations, although the effects were much stronger in Ad-CAng1 treated mice. Interestingly, vessel enlargement was not found in Tie1 deficient and Ad-CAng1 or Ad-Ang2 treated mice indicating that Tie1 is involved in angiopoietin induced tracheal vessel remodelling. Also, the venous markers Ephrin B4 (EbhB4) and P-selectin were decreased in the trachea of the Tie1 deficient mice after Ad-CAng1 or Ad-Ang2 treatment compared to control, where expression levels were strong in remodelling capillaries. Previously, the expression of venous markers induced in inflammation has been shown to increase after Ang1 stimulation (Fuxe et al., 2010). Also, we found that Tie1 silencing in

cultured ECs using shRNA lentivirus and *Tie1* deletion in mouse EC reduced proliferation after angiopoietin stimulation when compared to corresponding treatments in control, Tie1 expressing ECs *in vitro* and *in vivo.* These results indicate, for the first time, that Tie1 is necessary for angiopoietin induced EC proliferation leading to vessel enlargement and remodelling.

3. Angiopoietin induced Tie2 phosphorylation and Foxo1 inactivation is impaired in Tie1 deficiency (III)

Ad-CAng1, but not Ad-Ang2, induced the phosphorylation of Tie2 in lungs, as observed in Western blotting of lung lysates. Tie2 phosphorylation was less in *Tie1* deficient mice after Ad-CAng1 treatment. Also, Tie2 downstream signalling via the Akt serine kinase was less activated in *Tie1* deficient, Ad-CAng1 treated mice when compared to control, Ad-CAng1 treated mice. In *Tie1* silenced and Ang1 stimulated cultured ECs, Foxo1 was detected in nucleus indicating the activation of Foxo1, and a similar effect was detected *in vivo* in mouse trachea. Foxo1 was excluded from the nucleus and present in the cytoplasm in control, Ang1 -treated ECs and WT mice resulting in decreased levels of Foxo1 target gene expression including the endothelial cell specific molecule 1 (*Esm-1*) and *Ang2*, and this effect was less in *Tie1* deficient mice. Foxo1 target gene activation is associated with angiopoietin-induced vascular proliferation and enlargement in normal conditions, but the effect of Tie1 for Foxo1 activation has not been previously discussed. Transgenic expression of Ang2 in mouse ECs induced phosphorylation of Tie2 indicating that endogenous Ang2 is an agonist of Tie2. The differential effect of autocrine Ang2 and Ad-Ang2 on Tie2 activation may be due to various oligomeric forms of recombinant Ang2, the stoichiometry of the agonist Ang2 is not known. Also, Ang2 concentration and stimulation time may affect Ang2 activity as a Tie2 agonist (Teichert-Kuliszewska et al., 2001).

4. Ang2 acts as a Tie2 antagonist in inflammatory conditions (III)

To investigate Tie signalling in inflammation we treated mice with LPS or TNF-α. LPS treatment decreased tracheal Tie2 and Tie1 expression in mice. Tie2 was also decreased in the lung, as determined using Westerns blotting of lung lysates, resulting in decreased Tie2 and Akt phosphorylation and increased nuclear Foxo1 in tracheal vessels. Decreased Tie2 levels are in line with previous results (Mofarrahi et al., 2008). The levels of Ang2, which is a target of Foxo1 -mediated transcription, were increased in serum after LPS treatment. However, this did not induce Tie2 phosphorylation, demonstrating that Ang2 is an antagonist of Tie2 in inflammatory conditions. Previous studies have shown, that blocking Ang2 protects the vasculature in sepsis (Ziegler et al., 2013, Han et al., 2016).

5. Tie1 ectodomain is cleaved in inflammation leading to a loss of agonistic effects of Angiopoietins (III)

We also found the cleaved Tie1 ectodomain in mouse serum after LPS treatment. Interestingly, the sTie1 levels were also significantly increased in patients with acute Puumala hantavirus infection compared to healthy controls. TNF-α treatment also induced Tie1 cleavage *in vivo,* which is in line with Singh et al (2012) study, where they showed that Tie1 was cleaved after TNF-α treatment of cultured ECs. Ad-CAng1 treatment induced less Tie2 phosphorylation in the lungs after LPS administration of mice when compared to Ad-CAng1 treatment alone. Also, LPS treatment abolished Tie2 phosphorylation in the transgenic mice expressing Ang2 in ECs. These results support a concept that Tie1 ectodomain is required for the agonistic effects of angiopoietins for Tie2 activation. Interestingly, a 2-day treatment of mice with Ad-CAng1 before LPS administration, decreased the leakiness of blood vessels and circulating Ang2 levels and increased Tie1 levels in tracheal vessels and lung, suggesting that pre-treatment with Ang1 protected the vasculature via inhibiting the loss of Tie1 ectodomain under inflammatory conditions. These results are in line with Kim et al. (2016) and David et al. (2011) where they showed that Ang1 stabilized the endothelial barrier function and vascular leakiness in a murine model of Mycoplasma pulmonis and sepsis, respectivlely.

6. Direct interactions of Tie1 and Tie2 are induced by angiopoietins (III)

Tie1 does not bind angiopoietins but it has been reported to co-immunoprecipitate with Tie2, and Tie1 is also phosphorylated by Ang1 via Tie2 (Saharinen et al., 2005, Yuan et al., 2007). Ang2 induces significantly weaker Tie receptor activation when compared to Ang1, even though Ang2 also promotes Tie receptor localization to EC-EC junctions (Maisonpierre et al., 1997, Saharinen et al., 2008). Therefore, we decided to investigate how Tie1 is coupled to Tie2 signalling by analyzing direct Tie1 and Tie2 interactions, within a 10 nm distance, in intact cells after angiopoietin stimulation. For this we set up three different methods: 1. FRET based on acceptor photobleaching, 2. FLIM analyzed using frequency domain microscope and 3. TCSPC-FLIM. Three methods were used, since FRET based on acceptor photobleaching is affected by donor concentrations, creating possible bias, whereas TCSPC-FLIM directly measures photons emitted from an excited donor, thereby giving most exact information about the surrounding and the subcellular localization of the donor fluorochrome. I was able to set up TCSPC-FLIM technology at my department, after the purchase of the FLIM module with a pulsed laser source of illumination and its installment of the LSM 880 Airyscan.

6.1. FRET based on acceptor photobleaching

Hence, we hypothesized that Ang1 and Ang2 induce dissimilar Tie1-Tie2 interaction leading to different downstream signalling. As described above, our previous results

have shown that Tie1 and Tie2 co-localize in ECs following Ang1 or Ang2 stimulation (Saharinen et al., 2005, Holopainen et al., 2012). To analyze Tie1-Tie2 interaction at the molecular level, we set up FRET method based on acceptor photo bleaching that detects short distance (10 nm) molecular interactions. For FRET, we created ECs expressing membrane-bound Tie1 and Tie2 ectodomains that were intracellularly tagged with V5 and GFP respectively, considering the optimal length of the receptor cytoplasmic domains for FRET. ECs were treated with angiopoietins and V5 was detected using Cy3-conjucated anti V5 Ab. Using the FRET method, we showed a statistically significant FRET efficiency between Tie1 and Tie2 following Comp-Ang1 (29.1% ± 5.6%), Ang1 (29.2% ± 6.8%) and Ang2 (31.8% ± 5.6%) stimulations. In contrast, FRET efficiency in control untreated cells was 15.0% ± 6.4%.

To exclude any potential bias in the results due to variation in Tie1/Tie2 expression levels achieved via retroviral expression, we decided to measure the FRET efficiency from retroviral transfected HUVECs after FACS sorted according to Tie2 and Tie1 expression. HUVECs were sorted according to Tie expression to create cells with relatively uniform but low Tie2 expression levels and high Tie1 expression levels. High acceptor concentration is likely to increase weak FRET efficiency when acceptor photobleaching is used.

However, we found no difference in FRET efficiency following Comp-Ang1 (32.1% ± 5.4%), Ang1 (30.4% ± 2.7%) and Ang2 (29.2% ± 6.8%) stimulations of HUVECs where Tie receptor levels were adjusted, confirming our previous results. Again, FRET efficiency in control, untreated cells was 15.0% ± 6.4%. Tie1-V5 and a membrane-anchored GFP were used as a control FRET pair. No interaction was detected after angiopoietin stimulation between Tie1-V5 and the membrane-anchored GFP (FRET efficiency 3.3% ± 0.67%).

6.2. Frequence domain FLIM

We next used frequency domain FLIM to further verify our data, as this method is less sensitive to variations in donor expression level. In HUVECs sorted according to Tie receptor expression as above, the established GFP-lifetime (2.0 ns) was significantly decreased in EC-EC junctions in Comp-Ang1 (1.68 ns ± 0.09 ns), Ang1 (1.64 ns ± 0.07 ns) and Ang2 (1.65 ns ± 0.06 ns) stimulated cells compared to unstimulated cells (1.89 ns ± 0.07 ns). As a control, we used HUVECs expressing low level of Tie2 together with V5-tagged VE-cadherin. In Tie2-VE-cadherin transfected cells there were no significant differences in GFP-lifetimes between unstimulated (2.0 ± 0.1ns), Comp-Ang1 (1.95 ± 0.09ns), Ang1 (1.9 ± 0.09 ns) or Ang2 (1.88 ± 0.07 ns) ECs, indicating that Tie2 and VE-cadherin do not interact despite the localization of both proteins in cell-cell junction. As expected, with this method we were able to reduce the variation in the results.

6.3. Summary of FRET and FLIM results

The results using the FRET and FLIM indicate that the orphan Tie1 receptor directly interacts with Tie2 and this interaction is induced by angiopoietin stimulation,

although the angiopoietins only bind to Tie2. This interaction mostly occurs in the EC-EC junctions, although recombinant Ang1 induced also significant Tie1-Tie2 interaction in intracellular vesicles. These results are novel and appear to contrast some of the previously published results by Seegar et al. (2010), who reported using FRET based on acceptor photo bleaching that after Ang1 stimulation, Tie1 dissociates from Tie2. These studies were conducted using U2OS osteosarcoma tumor cells and EA.hy926 somatic endothelial-tumor hybrid cells, which may have affected Tie receptor trafficking when compared to our results from primary ECs (Seegar et al., 2010). Thus, our approach utilizes ECs, which are known to be the natural cell type of Tie receptor expression and also have well developed EC-EC junctions and EC specific junction proteins. Our results of the mode of Tie1-Tie2 interaction being induced both by Ang1 and Ang2 are in line with the *in vivo* results demonstrating that both ligands function as agonists in non-inflammatory conditions. The Tie1-Tie2 interaction was mediated by the Tie receptor ectodomains, which was rapidly cleaved from Tie1 in inflammation, correlating with reduced agonist activity of both Ang1 and Ang2.

7. Ang-Tie signalling is dependent on $\alpha_5\beta_1$-integrin (III)

In addition, we found that the Ang1-induced Tie2 activation and downstream signalling is dependent on β_1-integrin. Using the TCSPC-FLIM, we further investigated the molecular Tie1-Tie2 interactions in ECs where β_1-integrin was silenced using shRNA lentiviruses (shβ_1). We found that the Ang1-induced molecular interaction in EC-EC contacts between Tie1 and Tie2 was significantly decreased in shβ_1 ECs compared to control scramble silenced (shScr) ECs (Comp-Ang1 stimulated shScr 1.67 ns ± 0.09 ns vs. CAng1 stimulated shβ_1 1.73 ns ± 0.09 ns). Interestingly, translocation of endogenous Tie1 and Tie2 to EC-EC junctions was reduced in β_1-integrin silenced ECs, but not in Scr or β_3-integrin silenced ECs. Importantly, silencing of α_5-integrin or β_1-integrin, but not β_3-integrin, reduced the angiopoietin-induced Tie2 and Tie1 tyrosine phosphorylation, serine phosphorylation of the downstream Akt kinase, and nuclear exclusion of the Akt target Foxo1 transcription factor. The results demonstrating decreased interaction of Tie1 and Tie2 in shβ_1 ECs might reflect altered complex formation between Tie1 and Tie2 after angiopoietin stimulation. The FLIM/FRET method can detect protein-protein interactions that occur within 10 nm distance and in the absence of β_1-integrin, the Tie receptor complex may have a different structure. In contrast to silencing the β_1-integrin, blocking Abs that inhibit the binding of β_1-integrin to fibronectin did not affect Tie2 phosphorylation, suggesting a function for β_1-integrin distinct of its ECM binding. Furthermore, β_1-integrin silencing did not reduce VEGF-induced VEGFR2 phosphorylation. Our results are in line with and extend results by Cascone et al. (2005) who showed that Tie2, but not VEGFR-2, co-immunoprecipitated with $\alpha_5\beta_1$-integrin, and that Tie2 was not co-immunoprecipitated with $\alpha_2\beta_1$- or $\alpha_v\beta_3$-integrins. They also demonstrated that Ang1-Tie2 signalling was increased in ECs adherent to fibronectin matrix via $\alpha_5\beta_1$-integrin. In addition, we have shown that Ang2 can directly activate β_1-integrin resulting in stress fiber formation and subsequent EC destabilization (Hakanpaa et al.,

2015). Our results indicate for the first time that $\alpha_5\beta_1$-integrin promotes Ang1-induced heteromeric complex formation between Tie1 and Tie2, Tie receptor activation and downstream signalling. These results call for further studies to explore the function of β_1-integrin on angiopoietin-induced Tie receptor activation and angiopoietin-induced vascular processes *in vivo*.

8. Tie1 is required for Ang2 agonist function (III)

The function of Tie1 in angiopoietin signalling has remained one of the key questions in angiopoietin field that are poorly understood. Therefore, in this study (III) we investigated the effect of *Tie1* deletion on angiopoietin mediated Tie2 signalling and the requirement of Tie1 for the agonistic function of Ang2. As Ang2 is a weak Tie2 agonist *in vitro*, we used retrovirally transduced ECs expressing full length Tie2 fused via its intracellular domain to GFP (FL-Tie2-GFP). When stimulated with Ang2, Tie2 phosphorylation can be detected in these cells. shRNA silencing of *Tie1* decreased Tie2 phosphorylation after Ang2 stimulation when compared to control, shScr silenced cells. Interestingly, Tie2 was phosphorylated in the transgenic mice expressing Ang2 in ECs, but phosphorylation was not detected in the lungs of Ang2 transgenic mice in *Tie1* deleted background. These results demonstrated that Ang2 is a Tie2 agonist, in line with previous studies which showed that autocrine Ang2 acted as a weak Tie2 agonist and stabilized ECs in culture (Daly et al., 2006, Yuan et al., 2009). In addition, our results indicated that Tie1 is necessary for the Tie2 agonistic activity of autocrine Ang2 *in vivo* and contributes in a similar way in cultured ECs.

9. Tie2 trafficking is altered in the absence of Tie1 (III)

Angiopoietins induce Tie receptor translocation into EC-EC junctions (Fukuhara et al., 2008, Saharinen et al., 2008) and the reduced Tie2 phosphorylation observed upon *Tie1* silencing may be due to the impaired Tie2 trafficking in ECs. To investigate this possibility, we utilized time-lapse videomicroscopy of FL-Tie-GFP expressing HUVECs. We found that Tie1 silencing reduced the translocation of FL-Tie2-GFP into EC-EC junctions after Ang2 stimulation when compared to control, shScr silenced cells. Instead after Ang1 stimulation of *Tie1* silenced cells, some of the FL-Tie2-GFP was translocated into EC-EC junctions, but immediately internalized into early endosomes, also from all over the EC membrane. This was confirmed using Abs against the early endosome antigen 1 (EEA1). Notably, although *Tie1* silencing decreased Tie2 translocation into EC-EC junctions, no internalized vesicles were detected after Ang2 stimulation. In line with the results of increased Tie2 internalization after Ang1 stimulation of *Tie1*silenced ECs, Tie2 protein in the lungs of Ad-CAng1-treated, *Tie1* deleted mice was less compared to control, Ad-CAng1 treated mice.

Our findings are consistent with previous studies. Savant et al. (2015) demonstrated that after Ang1 stimulation of *Tie1* silenced HUVECs, Tie2 was internalized into lysosomes, detected by colocalization with the lysosomal associated membrane protein (Lamp-1), in. Also, the same authors demonstrated that prolonged Ang1

stimulation of *Tie1* silenced confluent ECs resulted in decreased Tie2 phosphorylation and decreased also the total Tie2 protein. Han et al. (2016) also demonstrated that Ang1, but not Ang2 induced Tie2 internalization via EEA1 positive endosomes. Interestingly, ABTAA, a monoclonal Ang2 Ab with therapeutic properties in murine sepsis models, induced strong Tie2 activation and internalization of Tie2. Our results further demonstrate that Tie1 is required for the agonist activity of both, Ang1 and Ang2 signalling, and that loss of Tie1 affects Tie2 trafficking after stimulation with both ligands. Interestingly, Tie2 mutations that cause venous malformations (VM) (Limaye et al., 2009) and primary congenital glaucoma (PCG) (Souma et al., 2016) have been reported to affect Tie2 trafficking in ECs. Limaye et al. (2009) demonstrated that overexpressed somatic L914F mutation of Tie2 in HUVECs was abnormally localized in endoplasmic reticulum (ER) in both unstimulated and Ang1 stimulated cells, and that Ang1 stimulation did not induce Tie2 translocation to the EC-EC junctions. Instead, the L914F VM associated Tie2 mutant was hyperphosphorylated in a ligand-independent manner. In addition, they found the same Tie2 L914F mutant in humans with sporadic VM. Also Souma et al. (2016) demonstrated that Tie2 mutants that cause PCG, do not translocate to EC-EC junctions after Ang1 stimulation of HUVECs. These results indicate that disease-causing mutations that either increase or decrease Tie2 activity can disturb Tie2 trafficking, demonstrating the importance of correct Tie2 trafficking in ECs for normal vascular responses *in vivo*. It would be interesting to investigate the potential role of Tie1 in the trafficking by the mutant Tie2 receptors.

10. Ang2 modulates endothelial cell-cell junctions in blood vasculature enhancing tumor cell extravasation and metastasis (IV)

Ang2 has been reported to be upregulated in many cancers and anti-angiogenic drugs targeting Ang2 are in clinical phase I-III trials. In this study (IV), we analyzed the effect of Ang2 in tumor metastasis *in vivo*, and the mechanisms of action of a therapeutic anti-Ang2 Ab *in vitro*. Transgenic mice expressing mouse Ang2 in the endothelium under doxycycline regulated promoter were used. In addition, subcutaneous syngeneic mouse melanoma (B16-F10) and lung carcinoma (LLC) models were used as well as human NCI-H460-LNM35 xenografts. Tumor growth, angiogenesis and lymphangiogenesis were analyzed, in addition to lung and lymphatic metastasis after systemically induced adenoviral Ang2 overexpression, as well as after administration of Ang2 blocking Abs.

Adeno-associated viral Ang2 delivery in mice for three weeks before implantation of tumor cells increased primary tumor growth of LNM35 tumor cells and elevated tumor lymphatic and blood vessel densities. Also, lymph node and lung metastasis were increased. Similar results were obtained using transgenic mice expressing Ang2 in ECs: Ang2 expression increased lung metastasis of intravenously injected tumor cells in a melanoma model when compared to control mice two weeks after injection. In immunodeficient mice, administration of therapeutic anti-Ang2 blocking Abs, but nor control Abs, reduced abnormalities of metastasis-associated lung capillaries, including

gaps between EC junctions and inhibited tumor metastasis, growth, angiogenesis and lymphangiogenesis.

To investigate the mechanism of action of the therapeutic anti-Ang2 Ab at the molecular level, we used BECs ectopically expressing of FL-Tie2-GFP via retrovirus. BECs secrete endogenous Ang2, resulting in the Tie2-Ang2 complex formation in EC-EC junctions. We showed that the anti-Ang2 Ab induced the internalization of Ang2-Tie2 complexes from EC-EC junctions resulting in decreased junctional Tie2 and Tie1 receptor localization. The anti-Ang2 Ab decreased Ang2-Tie2 complexes in EC junctions but did not affect the Ang1-Tie2 complexes, which are known to improve EC adherens junction integrity. Therefore, we investigated the integrity of the vascular endothelium in the lungs, where tumor cells had metastasized. We used the transgenic mice expressing Ang2 in ECs and WT mice after administration of anti-Ang2 blocking or control Abs and analyzed lung samples, using transmission electron microscopy. Interestingly, severely disrupted EC-EC junctions and EC detachment from the underlying BM were observed in Ang2 overexpressing, tumor cell injected mice compared to controls. Furthermore, the anti-Ang2 Ab treatment increased the integrity of the adherent EC-EC junctions in the lungs of wild-type mice, where tumor cells had extravasated. This was associated with reduced tumor metastasis. Our novel results suggest that Ang2 modulates EC-EC junctions, which enhances tumor cell extravasation. These effects can be prevented using anti-Ang2 Ab, which may lead to a stabilization of the EC-EC junction resulting in decreased tumor metastasis.

11. Ang2 in renal cell carcinoma (I-II)

RCC has an increased prevalence world-wide (Rini et al., 2009). RCC is resistant to chemotherapy and also, respond poorly to interferon and IL-2, which have been used in the treatment of RCC before targeted therapies (Rini et al., 2009, Coppin et al., 2011). Since 2005, seven targeted therapies have been approved for the treatment of mRCC, and as more therapies are coming available, including immune check-point inhibitors, understanding of the mechanisms of disease development and knowledge of patients that will benefit from these treatments are of great importance.

11.1. Ang2 is expressed in mRCC tumor endothelium and correlates with vascular density (I)

High circulating Ang2 has been reported to predict poor prognosis in metastatic breast and colorectal cancer and in melanoma (Sfiligoi et al., 2003, Helfrich et al., 2009, Goede et al., 2010), whereas the expression of Ang2 in the primary tumor tissue has not been widely investigated. One of the reasons may have been the lack of suitable methods, and few studies have demonstrated Ang2 expression in the neoplastic tumor cells. We developed an IHC-based method of detection of Ang2 in human RCC, and image based quantification of Ang2-positive tumor vasculature.

Thus, using IHC, we evaluated the expression of Ang2, CD31 and Ki-67 in clinical mRCC tumor samples, in a material containing 136 surgically removed primary tumors from mRCC patients who received first-line sunitinib treatment after the

surgery and follow-up data of the patients. In this project we investigated for the first time if the expression of Ang2 protein in the primary tumor has predictive value for sunitinib-treatment response in RCC. This is important because there is a need for the identification of biomarkers predicting the sunitinib response, but so far no such established markers are in clinical use (Ravaud and Schmidinger, 2013).

We found that Ang2 was exclusively expressed in the ECs of tumor blood vessels but not in tumor cells, and Ang2 expression in the vasculature correlated with vascular density as measured by CD31 IHC staining. Few studies demonstrate that high MVD in RCC is associated with more aggressive tumors, and predisposes to tumor metastasis (reviewed in Baluk et al., 2005) and also, predicts a poor prognosis in ccRCC (Joo et al., 2004, Yao et al., 2007). Instead, Virman et al. (2015) recently published that low expression of CD31 in RCC was associated with shorter OS. Li et al. (2013) demonstrated in their study using IHC that Ang2 is expressed in cytoplasm of oral squamous cell carcinoma. Currie et al. (2002) previously showed tumoral as well as endothelial expression of Ang2 using IHC in 45 RCC patient samples. In our study, we demonstrated the specificity of Ang2 IHC staining using recombinant Ang2 protein to neutralize the Ang2 Ab before IHC staining, which completely abolished the IHC signal. Initial scoring of the samples was based on vessel density in four categories (<5 Ang2 positive vessels/microscopic field, 5-50 Ang2 positive vessels/microscopic field, 50-100 Ang2 positive vessels/microscopic field and >100 Ang2 positive vessels/microscopic field). For statistical analysis, the samples were grouped to high (included 50 or more Ang2 positive vessels) and low group (included less than 50 Ang2 positive vessels in the microscopic field). High Ang2 group contained 38% of the samples, and low Ang2 group 62% of the samples. High CD31 group contained 60% of all samples, with 100 or more CD31 positive vessels per microscopic field, whereas the low CD31 group consisted of 40% of the samples. The segregation of samples based on CD31 IHC was based on a very clear cutoff of highly vascular mRCC tumors. High Ang2 expression level correlated with high CD31 expression level ($P < 0.0001$).

11.2. High Ang2 expression was correlated with better response to sunitinib treatment (I)

Best response to treatment, including both indicators of stable disease (SD) + PR was defined as CBR. When compared to low and high Ang2 expression, CBR was 91% in the high Ang2 group when compared to 76% in the low Ang2 expression group ($P = 0.033$). Only 9% of patients with high Ang2, compared to 24% of patients with low Ang2 expression had progressive disease (PD) as their best response to sunitinib treatment ($P = 0.033$). Also, similar results were observed in the CD31 groups: CBR was 94% in high and 74% in low CD31 expression groups ($P = 0.005$). In line with our results, Dornbusch et al. (2013) demonstrated that mRCC patients with high CD31 expression, had better response to sunitinib treatment. In our study, only 7% of patients with high CD31 expression, when compared to 25% of patients with low CD31 expression had PD as their best response ($P = 0.005$) to sunitinib treatment. Combination of high CD31 and high Ang2 expression predicted more frequently

clinical benefit with sunitinib (100% of patients with CBR) when compared to patients with low expression of both of the markers (76%) ($P = 0.002$). None of the patients with high CD31 and Ang2 expression levels had PD as their best response to treatment.

High pre-therapeutic Ang2 expression in the tumor vasculature was correlated with better response (PR/SD) to sunitinib treatment ($P = 0.03$). In our study, high expression of Ang2 or CD31 did not correlate significantly with PFS or OS. In line with these results, SD was not significantly different in patients with low levels of Ang2 and CD31 (median 10.5 months) compared to combination of high levels (median 8.7 months) ($P = 0.626$). Interestingly and as expected, our study showed significant correlation between clinical benefit (PR/SD) and longer PFS (14.1 months for median PFS for PR/SD patients vs. 3.3 months for PD patients, $P < 0.0001$) and also OS was significantly prolonged (30.1 months for PR/SD patients vs. 12.7 months for PD patients, $P < 0.0001$). Although high Ang2 and CD31 did not correlate with prolonged PFS and OS, they associated with CBR.

Recently published data from a clinical phase II trial (146 RCC patients) demonstrated that lower circulating Ang2 associated with better response to sunitinib (Motzer et al., 2014). However, the correlation between circulating and primary tumor Ang2 expression levels still remains to be investigated. One potential explanation for the differential results obtained using IHC and based on serum markers may be that Ang2 is stored in Weibel-Palade bodies of tumor vascular endothelium, which can be detected using IHC, and stored Ang2 level may not correlate with bioactive Ang2 levels. Stored Ang2 has been supposed to function in an autocrine manner in tumor endothelium, thus the correlation of tumor endothelial and circulating Ang2 level in human cancer needs more investigation. Also, differences in the levels of VEGF and VEGFR2 expression in tumors versus their circulating levels, and association of these parameters to response to sunitinib treatment have been reported in smaller studies previously. Terekawa et al. (2013) showed in their study including 40 mRCC patients that the expression level of VEGFR2 in the tumor tissue detected by IHC was significantly associated with PFS and response to sunitinib treatment, whereas Gruenwald et al. (2010) showed that soluble VEGFR2 failed to correlate with clinical response in sunitinib treated mRCC patients.

Although sunitinib treatment is known to prolong the median OS (Motzer et al., 2009b), complete durable responses are rare. This is associated with acquired resistance that develops during therapy. On the other hand, patients that do not benefit at all are considered to manifest with intrinsic sunitinib resistance. Giuliano et al. (2015) demonstrated *in vitro,* that sunitinib was internalized to the lysosomal vesicles (detecting with Lamp-1 Ab), but did not accumulate in early endosomes (detected with EEA1 Ab) in mRCC cells. Internalization of sunitinib reduced the activity of sunitinib resulting to a drug resistance. It has been proposed that Ang2 expression might induce resistance to anti-angiogenic therapies, based on mouse glioblastoma and breast cancer models (Scholz et al., 2015, Rigamonti et al., 2014). However, in our study high Ang2, as detected using primary tumor IHC, was associated with increased CBR

to sunitinib. Further studies are needed to investigate sunitinib resistance in ECs.

11.3. High Ki-67 expression in tumor cells predicted poor prognosis (I)

We used 10% Ki-67 positive nuclei as a cut off to categorize Ki-67 groups to high and low expression groups. We found a correlation between high pre-therapeutic expression of Ki-67 and worse outcome in patients treated with sunitinib. PFS was 6.5 months in patients with high Ki-67 when compared to 10.6 months in the low group (P = 0.009). In addition, a similar trend was observed in OS comparing high (15.7 months) and low (28.5 months) expression levels of Ki-67 (P = 0.015). Our results are in line with previous studies, where high Ki-67 expression in non-metastatic ccRCC was associated with poor patient prognosis (Tollefson et al., 2007, Gayed et al., 2014). However, our results are to our knowledge first to demonstrate predictive value for Ki-67 expression in mRCC, which has been treated with first-line sunitinib.

11.4. Angiogenesis and proliferation markers Ki-67 and BCL-2 as long-term prognostic factors in RCC patients (II)

To investigate the significance of Ang2 expression on the natural course of RCC, we used RCC samples from patients who received no targeted therapy. We evaluated Ang2 expression alone and together with cell proliferation and apoptosis markers (Ki-67 and BCL-2) and angiogenesis markers (VEGFR3 and CD31) and their effect on long-term survival of RCC patients. This study included 224 RCC patients who underwent nephrectomy between years 1985-1995 and were treated by same clinical practice. After this parallel TMAs were constructed for IHC analysis.

All samples were positive for endothelial Ang2 expression, and Ang2 was expressed only in ECs, which is in line with our previous study (I) (Rautiola et al., 2016). In this work we developed an image-based quantification method of digital IHC signal, and the results were expressed as percentage of Ang2 positive area. Expression of Ang2 positive area per total tissue area varied between 0.07 - 25.65%, and the median of Ang2 expression was 5.59%, which was used as a cut-off to categorize the samples into Ang2 high (>5%) and Ang2 low (≤5%) groups. Low Ang2 expression was detected 66.7% of the pRCC tumors, whereas high Ang2 expression was detected in 52% of the ccRCC tumors.

11.5. High Ang2 expression correlated with tumor grade, longer survival and low tumor cell proliferation (II)

High Ang2 expression was associated (P = 0.04) with tumor grade: 1-2 (62%), 3 (57%) and 4 (39%). Notably, very high Ang2 expression (top tertile, cut off 9.1% Ang2 positive area) correlated with longer survival. This is a very interesting result, challenging the current view that Ang2 promotes tumor progression, thereby predicting poor prognosis. As described before, and in our previous study (I) (Rautiola et al., 2016), tumor Ang2 may reflect Ang2 stored in endothelial Weibel-Palade bodies and may not correlate with the bioactive Ang2. However, it is interesting to speculate that Ang2 functions may be more complex, acting as an agonist in some tumors, and

as antagonist in others. The agonist activity might be regulated via tumor microenvironment associated factors, such as inflammation, and contribute to tumor vessel phenotype.

Ang2 expression did not correlate with CD31 expression, but was correlated with high VEGFR3 (P = 0.004). High Ang2 expression was also correlated with low Ki-67 (P = 0.032) and high BCL-2 (P = 0.012) expression. In univariate analysis, high Ki-67 expression combined with both, low or high Ang2 expression, showed shorter OS than low Ki-67 and high Ang2. Instead, low BCL-2 expression was associated with shorter OS when combined with low or high Ang2 expression than high BCL-2 and low Ang2. Multivariate analysis showed shorter OS of patients with high Ki-67 and low Ang2 expressions. Instead, combination of high Ang2, high Ki-67 and low BCL-2 predicted shorter OS. Virman et al. (2016) demonstrated using the same patient material that low BCL-2 and elevated Ki-67 were associated with poor prognosis. In addition, it has been demonstrated that RCC patients with high Ki-67 correlate with poor survival (Visapaa et al., 2003, Gayed et al., 2014). Also, our results are in line with previous results, demonstrating that low BCL-2 is associated with poor prognosis (Itoi et al., 2004, Kallio et al., 2004).

CONCLUSIONS

Current anti-angiogenic drugs, which inhibit the growth of new blood vessels, have revolutionized the treatment of many human cancers, however, complete responses are rare, and most patients are either refractory or relapse after a few months of treatment. Furthermore, no clear guidelines exist for patient- or tumor-specific selection of anti-angiogenic therapeutic agents, and so far no established biomarkers for anti-angiogenic therapies are in clinical use. Therefore, novel treatment modalities for cancer are urgently needed, as well as knowledge of the biomarkers for the selection of patients, who will benefit from the anti-angiogenic therapy. Angiopoietin-Tie system is a relevant and promising target for new anti-angiogenic therapies in cancer, but also, and perhaps more importantly in ocular neovascular eye diseases, and they also hold potential in many other vascular diseases, including inflammation/infection associated vascular leakage and diabetic vascular complications. Currently, the Ang2-targeted drugs are among the most advanced second-generation anti-angiogenic cancer therapies. However, the signalling mechanisms are poorly understood, and very little is known about Ang2 expression in human cancer.

This study investigated the Ang-Tie growth factor receptor system from the molecular level to the translational level in order to better understand the function of the Ang-Tie system in tumor angiogenesis and metastasis, and inflammation. Our results indicate that (1) orphan Tie1 is required for Ang-induced Tie receptor interaction, signalling, and Tie2 trafficking and (2) Tie1 is required for the agonist activity of Ang1 and Ang2 *in vivo* during vascular remodelling. We also demonstrated that (3) Ang-induced direct Tie1-Tie2 interaction in EC-EC contacts is dependent on β_1-integrin, and that β_1-integrin is required for Ang1 agonist activity. In addition, we showed that (4) anti-Ang2 Ab inhibits tumor growth and metastasis, and works at EC junctions to improve EC junction integrity during distant metastasis of the lungs. Moreover, we show that (5) the beneficial effects of the therapeutic anti-Ang2 Ab are likely mediated via internalization of Ang2-Tie2 protein complexes from the cell surface.

At the translational level, we show in that (6) high Ang2 expression in the tumor vasculature alone or when combined with high CD31 expression was associated with high CBR, but not PFS or OS, whereas low Ki-67 expression was significantly associated with prolonged PFS and OS in patients treated with first-line sunitinib (I). In another study (II) we show that (7) very high Ang2 expression in RCC tumor vasculature was associated with prolonged OS in patients who received no targeted therapies. This result is surprising, as serum Ang2 has been considered a marker of poor prognosis in many cancers.

Retrospective analysis of large patient cohorts from clinical trials would be informative to make conclusions about the potential of using Ang2 as a biomarker for anti-angiogenic or anti-Ang2 drugs. Furthermore, prospective analysis of large cohorts has the power to validate biomarkers identified in retrospective studies. Patient stratification based on clinical biomarkers should guide treatment decisions, and

thereby provide both clinical and economical benefit for the society. This research has uncovered several key questions about Ang-Tie biology and its function in the vascular endothelium, and shed light on the Ang-Tie expression in human cancer.

AKNOWLEDGEMENTS

This work was carried out in the Translational Cancer Biology Research Program at Biomedicum Helsinki, University of Helsinki.

This work was supported by grants from Doctoral Programme in Biomedicine (DPBM), Biomedicum Helsinki Foundation, Research Foundation of the University of Helsinki, Finnish Cultural Foundation, Ida Montin Foundation and University of Helsinki.

I want to thank my supervisor Docent Pipsa Saharinen for offering me the opportunity to do research in the environment with latest technologies and excellent lab facilities. I want to thank you for my really interesting project including all those challenging new methods that I set up with colleagues, enabling me to develop my skills as a researcher.

Professor Peppi Karppinen from University of Oulu, and Professor Tom Böhling from University of Helsinki, I am very grateful for reviewing and commenting critically my thesis. I wish to express my gratitude to Docent Anna Dimberg from University of Uppsala for accepting our invitation to be an opponent of my thesis. I acknowledge Professor Päivi Ojala for being a custodian in my thesis defence.

I wish to thank my thesis follow-up committee members Dr. Maria Vartiainen and Dr. Krister Wennerberg for the fruitful discussions and comments during our annual committee meetings, and also, I am really grateful for all of your support and help.

I want to thank all the collaborators during all these years; this work would not have been possible without you. I would like to especially thank Academy Professor Kari Alitalo, Docent and Chief of HUCH Cancer Center Petri Bono and Professor Pirkko-Liisa Kellokumpu-Lehtinen for the collaborative projects, which I was honoured to work with. For their collaboration, I also thank Dr. Ari Ristimäki, Professor Heikki Joensuu and Dr. Juha Virman. I wish to acknowledge Dr. Tuomas Mirtti for instruction of histology of renal cell carcinoma and help with the analysis and comments for our manuscripts. Thank you Dr. Juhana Rautiola for all numerous and humoral hours behind the microscope and hard work within a tight schedule. I would like to thank Biomedicum Imaging Unit and especially Jussi Kenkkilä for help to set up together the FLIM TCSPC method and all friendly support on imaging. I would like to thank also The Light Microscopy Unit at Viikki for all help and expertise with the early phases of the FLIM project.

All my dear past and present members in the Saharinen lab, I warmly thank all of your support and help as well as all the fun times in and outside lab hours. It has been wonderful to work with all of you. Kirsi Mänttäri, I'm highly thankful for your priceless assistance. In every busy day you always took time just to do "one more" experiment or staining. During the crappy days, you always made me smile and were my sunshine, I could not have done this project without you. Laura, I'm grateful for all the positiveness that you shared in the lab, and of course, I have no words how to thank you for your support in professional and personal life. Tuomas, I want to thank

you for our fruitful discussions during late office hours. Prson, I am grateful for the time we worked together, and specially your sense of humour, it is as bad as I have. The bad humour was our power!

I am very grateful to all wonderful people who worked around me. I would like to reserve a special thanks to Tapio Tainola for all your help in reassuring that all the reagents and facilities were the best to work with in the lab. You also took care that we had nice events and parties outside the lab. And of course, I would like to warmly thank you of our hard floorball games. I want to thank Emilia Korhonen for your collaboration and hard work days making our common manuscript as well as time outside the lab. Riikka, I like to thank you for all your help and advice in the lab and in the personal life. Kirsi Mattinen, I am thankful for all the nice events and rock concerts outside the office hours. I also want to thank that you have always been a trustable and great cat sitter for my lovely Doris cat. Maija, I warmly thank you for your friendship and all the support, and of course all numerous funny events, parties and evenings outside the office. I am happy that I have a friend like you. Thank you Krista, Jenny, Riitta, Tanja, Jaana, Katja, Leena, Irene, Pauliina and Vadim, I am grateful that I worked and had nice events with you.

I want to thank some special people and my friends outside the lab. My dear Lievestuore -girls Eija, Jenna and Tuire, I am thankful that you are in my life, and happy that after twenty years we have known each other, we still have our "girls' night out" evenings. Eija, I am grateful for your long-lasting friendship, and I've been blessed with the honor of being a godmother to your lovely son, Valtteri. Jenna, thank you for all your support, nice parties, summer festivals and all the numerous evenings and events, and all the discussions, when we have improved the way of life. You know, time will always help us. Tuire, you are the person I can always trust, and I am really grateful of all our events and fruitful discussions, which have been sometimes really serious, or not. My dear friend Katja, thank you for numerous times, you have saved my day and made me laugh just only by sending me a funny sms. I'm thankful for your friendship. I wish to thank Paula for all the nice, funny and humoral events and of course, the long trip we did together to Hongkong, Thailand, New Zealand and Australia. I wish to thank Eija Pirinen for all the encouragements and advice in professional and personal life. Hanna Heloterä, I am thankful for your friendship and I want to thank you for your support and many fruitful discussions. Jussi, Susanna and Mari, I'm grateful for all the liberating and relaxing evenings and events, and I want to thank you all for filling my free-time with laughter.

My love goes to my parents Alisa and Juhani, and my brother Petri, thank you for your love and support during this long and rocky road, I would not be here without you.

Without this PhD project, I would not have met my beloved Joonas; my other half, gym and travel buddy. I am grateful of your encouragement and patience during these busy years and all the funny and laughter events and times we have shared. I am looking forward to continue our Saturday morning squats at the gym, and of course, our trip through the life. Thank you for making me a lucky woman.

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